DIETARY INTAKE AND URINARY EXCRETION OF PHYTOESTROGENS IN RELATION TO CANCER AND CARDIOVASCULAR DISEASE

Michael Kent Reger

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Terrell W. Zollinger, DrPH, Chair

Doctoral Committee

Josette Jones, PhD

July 8, 2014

Ziyue Liu, PhD

Jianjun Zhang, MD, PhD

DEDICATION

To my parents, Ron and Peg Reger, for teaching me the value of education.

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Michael Kent Reger

DIETARY INTAKE AND URINARY EXCRETION OF PHYTOESTROGENS IN RELATION TO CANCER AND CARDIOVASCULAR DISEASE

Phytoestrogens that abound in soy products, legumes, and chickpeas can induce biologic responses in animals and humans due to structural similarity to 17β-estradiol. Although experimental studies suggest that phytoestrogen intake may alter the risk of cancer and cardiovascular disease, few epidemiologic studies have investigated this research question. This dissertation investigated the associations of intake of total and individual phytoestrogens and their urinary biomarkers with these chronic conditions using data previously collected from two US national cohort studies (NHANES and PLCO).

Utilizing NHANES data with urinary phytoestrogen concentrations and follow-up mortality, Cox proportional hazards regression (HR; 95% CI) were performed to evaluate the association between total cancer, cardiovascular disease, and all-cause mortality and urinary phytoestrogens. After adjustment for confounders, it was found that higher concentrations of lignans were associated with a reduced risk of death from cardiovascular disease (0.48; 0.24-0.97), whereas higher concentrations of isoflavones (2.14; 1.03-4.47) and daidzein (2.05; 1.02-4.11) were associated with an increased risk. A reduction in all-cause mortality was observed for elevated concentrations of lignans (0.65; 0.43-0.96) and enterolactone (0.65; 0.44-0.97).

Utilizing PLCO data and dietary phytoestrogens, Cox proportional hazards regression examined the associations between dietary phytoestrogens and the risk of prostate cancer

incidence. After adjustment for confounders, a positive association was found between dietary intake of isoflavones (1.58; 1.11-2.24), genistein (1.42; 1.02-1.98), daidzein (1.62; 1.13-2.32), and glycitein (1.53; 1.09-2.15) and the risk of advanced prostate cancer. Conversely, an inverse association existed between dietary intake of genistein and the risk of non-advanced prostate cancer (0.88; 0.78-0.99) and total prostate cancer (0.90; 0.81-1.00).

C-reactive protein (CRP) concentration levels rise in response to inflammation and higher levels are a risk factor for some cancers and cardiovascular disease reported in epidemiologic studies. Logistic regression performed on NHANES data evaluated the association between CRP and urinary phytoestrogen concentrations. Higher concentrations of total and individual phytoestrogens were associated with lower concentrations of CRP.

In summary, dietary intake of some phytoestrogens significantly modulates prostate cancer risk and cardiovascular disease mortality. It is possible that these associations may be in part mediated through the influence of phytoestrogen intake on circulating levels of C-reactive protein.

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General Introduction

Distribution and Burden of Cancer and Cardiovascular Disease

Cardiovascular disease and cancer are the two leading causes of death in the United States (1) and many other developed countries throughout the world (2). In 2010 in the US there were a total of 597,689 deaths due to cardiovascular disease and 574,743 deaths due to all types of cancer. These two types of chronic diseases accounted for 47.5% of the total deaths in the US in 2010 (1). On a global scale, cardiovascular disease is the leading cause of death worldwide, estimated to account for 13.2 million deaths in 2011 (2). Total cancer claimed an estimated 8.2 million lives worldwide in 2012 (3). Cancers of the lung and bronchus are the most common incident forms of cancer as well as the leading cause of cancer death in the US, accounting for an estimated 224,210 new cases and 159,260 deaths in 2014 (4). Prostate cancer is the leading form of incident cancer (estimated 233,000 cases in 2014) and second leading cause of death (estimated 29,480 deaths in 2014) among American men (4).

The causes of prostate cancer are not yet completely understood, however, past studies have established several risk factors that may increase the risk of prostate cancer development. Most of the risk factors that have been established are non-modifiable, such as age (prostate cancer risk rises rapidly after the age of 50, and nearly two out of three cases are found in men over the age of 65), race/ethnicity (African-Americans are more likely to develop and twice as likely to die from prostate cancer), and family history

of disease (having a first degree relative with prostate cancer doubles the risk of prostate cancer) (4). Evidence has also suggested that there are several genes that may cause an increased risk of prostate cancer and account for between 5% and 10% of the total cases. Genetic mutations in the Hereditary Prostate Cancer Gene 1 (HPC1), the androgen receptor, and the vitamin D receptor have all been linked to an increased risk of prostate cancer (5). Modifiable risk factors such as obesity (6), smoking (7), consuming higher amounts of red meat and lower fruits and vegetables (8), or lower levels of vitamin D (9) have also been associated with an increased risk of prostate cancer incidence or mortality.

Factors that increase the risk of cardiovascular disease, both non-modifiable and modifiable, are more established than risk factors for prostate cancer. Non-modifiable risk factors that increase the risk of cardiovascular disease include age (approximately 82% of cardiovascular disease deaths occur after the age of 65), gender (men are at a greater risk than women), and race (African-Americans) (10). In addition to these non-modifiable factors, a global case-control study across 52 countries established several modifiable risk factors regardless of race and gender. The modifiable risk factors with the greatest increased risk of cardiovascular disease included being a current smoker (OR: 2.87; 95% CI: 2.58, 3.19), psychosocial factors (OR: 2.67; 95% CI: 2.21, 3.22), history of hypertension (OR: 1.91; 95% CI: 1.74, 2.10) and abdominal obesity (OR: 1.62; 95% CI: 1.45, 1.80). This study also identified several factors that decrease the risk of cardiovascular disease, including daily consumption of fruits and vegetables (OR: 0.70; 95% CI: 0.62, 0.79) and regular physical activity (OR: 0.86; 95% CI: 0.76, 0.97) (11).

Although several risk factors have been established for both prostate cancer and cardiovascular disease, it is important to continue to identify modifiable factors that may

decrease the burden of these diseases in the US and on a global scale. One such modifiable factor that has thus far not been definitively explored is diet, particularly the intake of phytoestrogens and the resulting influence on the incidence and mortality of both prostate cancer and cardiovascular disease. Phytoestrogens are a group of non-steroidal plant metabolites that are structurally similar to 17β-estradiol and therefore have the ability to bind to estrogen receptors (12). The main forms of phytoestrogens and their metabolites are briefly reviewed below.

Sources and Biochemistry of Estrogen and Phytoestrogens

Estrogen

Estrogens are a group of compounds that are named for their importance in both the menstrual and estrous reproductive cycles. Natural estrogens are steroid hormones and are the primary female sex hormone (13). Like other hormones of the steroid class, estrogens diffuse across the cell membrane, and upon entering the interior of the cell, bind to and activate estrogen receptors. Activation of estrogen receptors modulates the expression of many genes (14).

The three main forms of naturally occurring estrogens in women are estrone (E1), estradiol (E2), and estriol (E3). Estradiol is the most predominant estrogen during a woman's reproductive years in terms of both the absolute level found in the serum as well as in terms of estrogenic activity (15). Estriol is the most plentiful of the three estrogens but also the weakest, with estradiol showing a potency of approximately 80 times that of estriol (16). Thus, estradiol is the most important of the naturally occurring estrogen hormones in females who are between the stages of menarche and menopause.

An exception to this rule occurs during pregnancy when the most important role shifts to estriol. In post-menopausal women, estrone becomes the primary form of estrogen found in the body (17).

Estrogens in non-pregnant females are produced primarily by the ovaries. Follicle stimulating hormone (FSH) stimulates the production of estrogens in the ovaries by the granulosa cells of the ovarian follicles and corpora lutea. Some forms of estrogens are produced in smaller amounts by other tissues in the female body including the liver, adrenal glands, and the breasts. Estrogens produced from these secondary sources are most important in post-menopausal women (18).

The actions that result from estrogen are mediated by the estrogen receptor (ER). The estrogen receptor is a dimeric nuclear protein that binds to DNA and controls gene expression. The estrogen-receptor complex can then bind to specific DNA sequences to activate the transcription of target genes. Since estrogen has the ability to enter all cells through passive diffusion across the cell membrane, its actions are dependent on the presence and availability of the estrogen receptors located within the cells. The estrogen receptors are expressed mainly in the specific tissues of the ovary, uterus, and breast (19).

In addition to the activation of genes, estrogen serves many additional purposes in the human body, mostly in females. Some of the additional functions include:

- Structural
    - Promote development of female secondary sex characteristics (20)
    - Accelerate metabolism (21)
    - Stimulate endometrial growth (22)
    - Increase uterine growth (23)

    - Reduce bone resorption, and therefore increase bone formation (24)
- Protein synthesis
    - Increase the hepatic production of binding proteins (25)
- Coagulation
    - Decreases platelet aggregation (26)
    - Decrease antithrombin III (27)
- Lipid
    - Increase HDL, triglyceride (28)
    - Decrease LDL, fat deposition (21)
- Fluid balance
    - Sodium and water retention (29)
- Gastrointestinal tract
    - Reduce bowel motility (30)
- Uterine lining
    - Together with progesterone, estrogen promotes and maintains the uterine lining in preparation for implantation of a fertilized egg (31)
- Ovulation
    - A surge of estrogen induces the release of luteinizing hormone which triggers ovulation (32)

Phytoestrogens

Phytoestrogens are naturally occurring plant compounds that are both structurally and functionally similar to the estrogen hormone found in mammals. Most phytoestrogens are phenolic compounds of which isoflavones are the most widely researched metabolites. Isoflavones are found in a wide variety of foods, including berries, wine, grains, and nuts but are most abundant in soybeans and therefore a wide variety of soy products (33). The common biological roles of phytoestrogens are to protect plants from stress as well as to act as part of the plants defensive mechanisms (34). Some ecologists have speculated that phytoestrogens in plants may have evolved to protect the plants by interfering with the reproductive ability of grazing animals (35).

Phytoestrogens have the ability to cause estrogenic and/or antiestrogenic effects in mammals due to their structural similarity to the hormone 17β-estradiol. In general, phytoestrogens are relatively weak estrogens, requiring a much higher concentration than their human hormone counterpart in order to produce an equivalent biological response. Since isoflavones are found in a wide variety of foods regularly consumed by humans, this class has generated the most interest in recent studies (36).

Phytoestrogens exert their effects primarily through binding to estrogen receptors in the human body. There are two variants of the estrogen receptor (ER), alpha (ER-α) and beta (ER-β). Many phytoestrogens have displayed a higher affinity for the beta estrogen receptor compared to the alpha estrogen receptor (37). There are several key structural elements that enable phytoestrogens to bind with a high affinity to estrogen receptors and therefore display effects that are similar to the human hormone, these key structural elements include:

- A phenolic ring that is necessary for binding to an estrogen receptor
- The ring of isoflavones that mimics a ring of estrogens at the estrogen receptor binding site
- A low molecular weight that is very similar to the human hormone estrogen
- The distance between two hydroxyl groups at the isoflavones nucleus that is similar to that occurring in estradiol
- Optimal hydroxylation pattern (36)

The main feature of the chemical structure of phytoestrogens that allows for binding to the estrogen receptor is the presence of the phenolic ring. The presence of this structure allows phytoestrogens to act as weak estrogen agonists, partial agonists, or as antagonists to endogenous estrogens such as estradiol at the estrogen receptor complex. Therefore, mimicking the endogenous estrogen, phytoestrogens may have similar effects as estrogen, or block the effects of estrogen (38). Both circulating and urinary biomarkers have been developed to assess phytoestrogen intake.

Blood tests: Phytoestrogens persist in the plasma for approximately 24 hours after ingestion. The plasma half-life for genistein and daidzein is approximately 8 hours in adults with peak measurable concentration occurring 6-8 hours after ingestion. Adherence to a high soy containing diet will lead to a high steady state of phytoestrogen concentration in the plasma. Adults consuming approximately 50 mg per day of total isoflavones will show similar plasma concentrations to those of Japanese citizens who consume a higher volume of soy products in their traditional diet (39).

Urine tests: Most studies attempting to observe phytoestrogen intake have focused on urinary excretion as a more effective method of measurement. The main reason for the

tendency to focus on urinary output of phytoestrogens is because of the high concentrations that can be found in the urine after soy intake that allows for a more accurate measure of the total intake of phytoestrogens. Nearly all of isoflavones are excreted in both the urine and feces within 24 hours after ingestion (40). There is a considerable variation between individuals in bacterial metabolism of daidzein in the gut which leads to markedly different urinary concentrations of phytoestrogens in different individuals (41). In NHANES 2001-2002, the mean urine concentration for daidzein in the total population ages 6 and older was 48.6 µg/L with a range from the 50th percentile to the 95th percentile of 48.5 to 957.0 µg/L (42).

Isoflavones

Isoflavones are a group of phytoestrogens most commonly found in foods that are consumed by humans. Isoflavones are found in abundance in soy products, legumes, and chick peas (43). The three most prevalent isoflavones that are present in plant based foods are genistein, daidzein, and glycitein (36), however the two most studied are genistein and daidzein.

Isoflavones are a subgroup of flavonoids. Flavonoids are the most common group of polyphenolic (organic chemicals that are characterized by the presence of large multiples of phenol structural units [C6H5OH – a hydroxyl group that is found on a benzene ring]) compounds that are consumed in the human diet and are found exclusively in plants (44). The widespread distribution of flavonoids along with their low toxicity compared to other plant compounds means that humans ingest significant quantities in their regular diet. In

vitro studies have shown that flavonoids may have anti-allergic, anti-inflammatory (45), anti-microbial (46, 47), and anti-cancer (48) effects on the human body.

Genistein

Genistein is a phytoestrogen that belongs to the category of isoflavones. It binds to estrogen receptor and exhibits weak estrogenic and weak anti-estrogenic effects (49). Genistein also binds to and inhibits protein-tyrosine kinase, which disrupts signal transduction and induces cell differentiation. It also inhibits topoisomerase-II which leads to DNA fragmentation and apoptosis (50).

Exposure to genistein occurs by food intake, principally through foods made with soybeans and soy protein. Isoflavones, such as genistein, found in soy products are contained in two chemical forms, aglycones (the unconjugated form) and glucosides (which are bound to a sugar molecule). The main dietary source of genistein is the biologically active glucoside genistin. Digestion or fermentation of soybeans, soy protein, or other soy products results in the release of a sugar molecule from the isoflavone glycoside, genistin, which results in the isoflavone aglycone, genistein (40). Before genistein can act it first must be released from genistin through the release of sugar. This process normally occurs in the stomach through acid hydrolysis or the intestine through the action of bacterial enzymes (40). There is considerable variation in the absorption and metabolism of genistein (49).

Genistein can affect the process by which signals at the surface of the cells are transferred to the interior of the cell. Genistein also inhibits the activity of several

enzymes that are important for controlling cell growth and regulations. The full effect of genistein exposure to the human body is still unknown (51).

Exposure to genistein can be measured by either a blood or urine test; however levels vary greatly due to individual variability of the metabolism of genistein (49). Despite the individual variability that has been found, studies have shown that the mean concentration of genistein shown through biological measurements in the blood or the urine does not differ greatly between men and women. A study of 1,414 adults from nine countries in Europe found that the mean concentration of genistein that was found in the plasma of men in the study was 1.77 mg/L and the mean concentration in the plasma for women was 1.70 mg/L (52). In the 2001-2002 National Health and Nutrition Examination Survey (NHANES), males and females also had similar levels of genistein measured in their urine output; the mean urinary concentration for males was 32.2 µg/L and the mean concentration for females was 33.7 µg/L (42).

Daidzein

Daidzein belongs to the isoflavone class of phytoestrogens. It binds to estrogen receptors and exhibits weak estrogenic and weak anti-estrogenic effects. Exposure to daidzein occurs by food intake, principally through foods made from soybeans or containing soy protein. Like genistein, daidzein is contained in soy products in two chemical forms, aglycones (unconjugated form) and glucosides (which are bound to a sugar molecule). The main dietary source of daidzein is the biologically active glucoside daidzin. Like with genistein, fermentation or digestion of soy products results in the release of the sugar molecule from the glucoside, daidzin, which results in the formation

of the other form, the aglycone daidzein (40). This process normally occurs in the stomach through acid hydrolysis and in the intestine through the action of the bacterial enzymes (40).

In a small proportion of the population, daidzein can be metabolized by the bacteria located in the intestine to produce metabolites that have a stronger estrogenic effect than daidzein itself. These metabolites are equol and O-desmethylangolensin (O-DMA). Daidzein has the ability to cross the placenta and has been found in breast milk as well (38)

Exposure to daidzein can be measured by either a blood or urine test; however levels of daidzein found in the blood or urine vary widely by person due to the variability of an individual's metabolism (38). Tests have shown however that the mean concentration of daidzein found in either the blood or the urine does not differ significantly between men and women. A study of 1,414 adults from nine countries in Europe found that the mean concentration of daidzein in the plasma for men was 0.89 mg/L and the mean concentration in the plasma for women was 0.80 mg/L (52). In the 2001-2002 NHANES, males and females also had similar levels of daidzein measured in the urine output; the mean urinary concentration for males was 49.8 µg/L and the mean concentration for females was 53.6 µg/L (42).

Glycitein

Glycitein belongs to the isoflavone class of phytoestrogens. It accounts for only 5% to 10% of the total isoflavones that are found in soy food products, but may be as high as 40% in some soy supplements composed mainly of soy germ. The estrogenic effect of

glycitein is considerably weaker than the effects observed by the other soy isoflavones, genistein and daidzein. Because glycitein has a structure that is similar to both genistein and daidzein, it may be important to evaluate the estrogenic activity of this isoflavone as well, despite accounting for a relatively low proportion of the total isoflavone content found in most foods (53).

Coumestrol, Formononetin, and Biochanin A

Three phytoestrogens, coumestrol, formononetin, and biochanin A, belonging to the class of isoflavones, are found to a lesser degree in soy products, legumes, and chick peas (54). Although there is a decreased concentration of these isoflavones in dietary products, they still have biologically significant properties that may protect against specific cancers or cardiovascular disease. Coumestrol has been identified as a novel reversible ATP competitive casein kinase 2 (CK2) inhibitor (55). CK2 is involved in various cellular events that include proliferation (56), apoptosis (57), and RNA synthesis (58). Overexpression of this protein kinase is associated with multiple types of cancer in humans, including cancer of the colon (59), prostate (60), and breast (61). Through the inhibition of CK2 expression, coumestrol has been shown to inhibit the growth of and trigger apoptosis in cancer cells (55).

Formononetin has been identified to have a possible therapeutic effect on acute myocardial infarction. Zhang et al. conducted an experiment on male Sprague-Dawley rats in which myocardial infarction was induced. The rats were randomized to treatment groups of different concentrations of a water-soluble derivative of formononetin or a control group. The rats receiving treatment after myocardial infarction showed a number

of significant improvements over the untreated group, including a reduced myocardium necrosis score, increased cardiac mitochondrial ATP content, and improved ATP synthase activity. The authors concluded from these findings that this derivative of formononetin has a protective potential against myocardial infarction injury (62).

Biochanin A has been identified as having possible chemopreventive actions against specific types of cancer (63). One of the mechanisms for cancer prevention by Biochanin A is through induction of sulfotransferases (64), which are a family of drug metabolizing enzymes which are important for detoxification (65) and regulation of biological metabolism enzyme genes (66). Improper regulation of sulfotransferases may lead to improper functioning of biological signaling molecules, which may result in cancer or other diseases (65). In an experiment conducted by Chen et al., the authors demonstrated that treatment with Biochanin A in rats can induce the expression of sulfotransferases, thereby promoting proper signaling and preventing certain chronic conditions (64).

Lignans

Lignans are another class of phytoestrogens that are found in plants. Plant lignans can be metabolized by the intestinal bacteria to form mammalian lignans, enterodiol and enterolactone (67). Flax seed and sesame seed contain the highest levels of lignans in food consumed by humans, however other sources include cereals, soybeans, cruciferous vegetables, and some fruits (68). One study of common plant foods discovered that flaxseed flour and its defatted meal produced the highest yield of enterodiol and enterolactone, up to 800 times higher than other plants (69). Considering the food

sources for both lignans and isoflavones, lignans are considered to be the principal source of dietary phytoestrogens in typical Western diets (70).

Like isoflavones, lignans are also polyphenols. Since lignans are metabolized by the intestinal bacteria into enterodiol and enterolactone, concentrations of these phytoestrogens measured in the serum or urine reflect both the dietary intake and the activity level of the intestinal bacteria (71). Studies have shown that lignans may exhibit estrogenic or anti-estrogenic (72), anti-oxidative (73), cardioprotective (74), and anti-cancer (75) effects on the human body.

Enterodiol and Enterolactone

Enterodiol and enterolactone are known as enterolignans and are converted from plant lignans in food consumed by humans in the upper part of the of the intestine by aerobic microflora (76). There are several steps that occur during the conversion from plant lignans to enterolignans, including hydrolysis, dehydroxylation, and demethylation. This process converts plant lignans to enterodiol, which can be then oxidized to form enterolactone. The conversion of enterodiol to enterolactone depends on the precursor and varies from less than 15% to 100% (77). Enterodiol and enterolactone can be measured in both the plasma and urine of humans (78). Epidemiologic studies have shown that both enterodiol and enterolactone may have preventive effects on such diseases such as cardiovascular diseases (79) and specific types of cancer (80, 81).

Current Experimental and Epidemiologic Evidence

Phytoestrogens, Total Cancer, and Prostate Cancer

Biologic Effects

The majority of studies on the association between phytoestrogens and prostate cancer have consisted of experimental studies examining the biologic effects of the different phytoestrogen metabolites. These types of studies include animal models, in-vivo studies, and gene-studies.

Animal Models

Relevant studies on the effects of phytoestrogens on prostate cancer in animal models consisted of observing the effects on tumors in mice or rats, both in vitro and in vivo. Most studies focused on the effects of genistein while one examined the effects of daidzein.

Three relevant published manuscripts observed the effects of genistein on animal models. The first study, conducted by Miekus and Madeja, investigated the effect of genistein on the proliferation and migration of rat prostatic carcinoma, with the hypothesis that genistein consumption reduces the incidence of metastases. Observing the cells in vitro, the authors concluded that at physiological relevant concentrations, genistein inhibited the motility of prostate cancer cells and therefore showed anti-metastatic activity, which supported the hypothesis. The authors' concluded that although it appeared that genistein reduces the incidence of metastases in vitro, the in vivo mechanisms may be more complex (82).

The second study, conducted by Liss et al., observed the differences in genes that are differentially regulated in prostate cancer cells treated with purified genistein or other soy protein isolates. The objective of this study was to discover if genistein was the major contributor to the anti-cancer effects of soy products seen in other studies, or if all of the metabolites found in soy products made contributions to this effect. In this in vitro study of both rat and human prostate cancer cell lines, there were several observations, including: 1) soy was more effective than purified genistein on decreasing cancer cell growth in certain prostate cancer cell lines, but the dose-related effect on cell proliferation was stronger in purified genistein; 2) there was no difference between soy and purified genistein on the influence of the steroid pathway; 3) of the eight genes of interest in the apoptosis pathway, there was a single gene that was up-regulated by purified genistein but not soy; 4) gene expression in cell cycle pathways showed similar results from soy and purified genistein; and 5) of the nine genes of interest in the nuclear receptors, there was a single gene that was up-regulated from the treatment with purified genistein but not soy. From these observations the authors concluded that genistein is likely the major contributor to the effect of soy on prostate cancer cells, however the differences in gene expression suggest that there is some complexity in the different compounds found in soy proteins (83).

The third study on genistein conducted by Nakamura et al. examined the effects of genistein treatment on advanced stage prostate cancer tumors in white mice in vivo. Observations in this study included an increased rate of lymph node and secondary organ metastases in the genistein treated white mice compared to the untreated control group and cancerous cells in the treated group showed more proliferating cells and fewer

apoptotic cells than the untreated group. These results that showed genistein treatment might increase the risk of prostate cancer could be due to the cancerous cell-line that was selected. Genistein is known to have a high binding affinity to estrogen-receptor β and the cell line that was chosen for this experiment had exclusive expression of this type of receptor. These observations generated the hypothesis that the tumor stimulatory effects are mediated by estrogen-receptor beta activation. The authors concluded that careful selection needs to be made when enrolling patients for treatment with genistein as certain cell lines or patients may have differential estrogen-receptor beta expression (84).

The final relevant animal model study was conducted by Singh-Gupta et al. and observed the effect of daidzein on hormone refractory prostate cancer both in vitro and in vivo. This study was designed after evidence emerged that pure genistein promoted increased metastasis to lymph nodes and secondary organs (84). The authors hypothesized that daidzein would negate the effects of genistein-induced metastases. The most relevant observations made in this study included: 1) daidzein protects against genistein-induced lymph node metastasis, shown by treating prostate tumors with a natural formulation of soy isoflavones compared with pure genistein; and 2) daidzein alone is less effective than pure genistein or a natural formulation of soy in killing cancerous cells. These results led to the conclusion that the treatment of prostate cancer with pure genistein should not be recommended as it appears that daidzein is important to suppress the metastatic effects (85).

In-Vitro Studies

In vitro studies on the effects of phytoestrogens on prostate cancer have been much more extensive. Most of these types of studies were attempting to observe a change in a specific mechanism within the cancer cell when exposed to phytoestrogens, while others examined the combination of phytoestrogens and other forms of treatment.

Hsieh and Wu observed, like many others, that Asian men exhibit a lower incidence of hormone-refractory prostate cancer (HRPC) compared to American men. Furthermore, Asian men who move to the United States and adopt an American lifestyle and diet show rates of HRPC that is indistinguishable from Caucasian men. These findings suggest that the diet in Asia contains certain ingredients that are protective against prostate cancer. With this observation, these researchers investigated if a combination of epigallocatechin gallate (EGCG), genistein, and quercetin (commonly found in the Asian diet) may synergize to control proliferation and gene expression in prostate cancer cells. Using an in vitro model with the addition of these phytochemicals, Hsieh and Wu observed a suppressed proliferation of the cancer cells, as well as a synergy in the expression of the androgen receptor, tumor suppressor p53, and detoxification enzyme reductase type 1 (NQO1). From these observations the authors concluded that there was feasibility for the development of a diet-based approach for prostate cancer prevention and treatment (86).

A study conducted by Oh et al. investigated if a combination of the lowering of cholesterol and genistein treatment were related to prostate cancer cell survival. In this study, this combination had several effects on the prostate cancer cells, including: 1) decreasing the protein expression of prostate Akt as well as the androgen receptors

stimulated by EGF and DHT in concentration dependent manners; and 2) inhibiting the phosphorylation cascade of Akt, GSK-3beta, and p70S6K. Both of these effects will ultimately inhibit the viability of prostate cancer cells and finally result in increased apoptosis. These results suggested that prostate cancer cells could be effectively inhibited by the combination of lowering cholesterol and the addition of genistein to the diet (87).

A study conducted by Swami et al. observed that soy and the isoflavone genistein inhibited the development and progression of prostate cancer through the inhibition of prostaglandins, which are known stimulators of prostate cancer growth. In prostate cancer cell cultures, genistein reduced the secretion of prostaglandins as well the prostaglandin receptor mRNA, thereby demonstrating two mechanisms for the prostaglandin biological effects. This study also included a small, randomized double-blind clinical trial where patients were given a placebo or soy isoflavone supplements prior to prostatectomy. In the treatment group, the patients showed a decrease in receptor mRNA leading to the conclusion that soy isoflavone supplements may be beneficial to prostate cancer chemoprevention and treatment (88).

A study conducted by Xu et al. investigated the target for genistein in prostate cancer cells in six different prostate cancer cell lines. All six cell lines showed that genistein bound to mitogen-activated protein kinase 4 (MEK4) causing an inhibition and a subsequent decreased expression of matrix metalloproteinase-2 (MMP-2). The MMP-2 level in untreated prostate epithelial cells was statistically significantly higher than the treated group by 100%. These observations increased the knowledge of the genistein mechanism in prostate cancer cells by leading to the conclusion that MEK4 was the target

for genistein in prostate cancer cells and was associated with decreased levels of MMP-2 (89).

A study conducted by Yuan-Jing et al. demonstrated that genistein induces apoptosis in prostate cancer cells. In this study the observation was made that the addition of genistein to certain prostate cancer cell lines in vitro demonstrated an increased induction of apoptosis in prostate cancer cells. Additionally, genistein did not increase apoptosis in normal cells. These results were observed both with and without the presence of survivin, which inhibits the apoptosis protein in cancer cells allowing them to continue to grow and proliferate (90).

Lee et al. investigated if genistein treatment was involved in the regulation of insulin-like growth factor (IGF-1). Elevated levels of IGF-1 are associated with increased risk of several different types of cancer (including prostate). The inhibition of IGF-1 and the downstream signaling pathways mediated by the activation of the IGF-1 receptor may be involved in inhibiting prostate carcinogenesis. In this study, genistein treatment decreased the number of IGF-1 stimulated cells and therefore caused a significant inhibition of cell growth that is stimulated by IGF-1. It was also observed that in treated cells genistein inhibited the phosphorylation of the IGF-1 receptor and the downstream targets. These results showed that genistein treatment effectively inhibited cell growth in IGF-1 stimulated prostate cancer cells, another mechanism by which genistein treatment may be effective in the treatment of prostate cancer (91).

A study conducted by Zhang et al. investigated if treatment with genistein had an effect on prostate cancer stem cells. Prostate cancer stem cells are involved in both tumorigenesis and progression of prostate cancer. Conventional cancer treatments often

fail to eradicate these types of cells, which can often lead to relapse, therefore targeting of prostate cancer stem cells may be an important treatment strategy. Prostate cancer tumorsphere cells possess some of the same qualities as prostate cancer stem cells. In this study, genistein inhibited tumorsphere formation, growth of tumorsphere cells, and colony formation of prostate cancer cells. These observations demonstrated that genistein may be a dietary phytochemical with the potential to target prostate cancer stem cells and therefore limit recurrence (92).

A study conducted by Adam et al. investigated if the inclusion of dietary phytochemicals in combination with an oncolytic adenoviral mutant that has been engineered to selectively target tumors with deregulated cell cycle and apoptosis pathways could interact synergistically to produce better results. This study combined an oncolytic adenoviral mutant (AdΔΔ) with genistein and other phytochemicals to observe the synergistic effects. This study observed synergistic and increased cell killing in prostate cancer cells when genistein and the oncolytic adenoviral mutant were used in combination compared with the phytochemicals alone. In addition, in vivo studies of this combination showed reduced tumor growth without toxicity in normal tissue. These findings suggest that the use of phytoestrogens in combination with other treatment types might be a feasible anti-cancer strategy (93).

Gene-Studies

Other studies have investigated the effects of phytoestrogens (mainly genistein) on specific prostate cancer genes. Evidence from these gene studies show that phytoestrogens affect many genes in different ways.

A study conducted by Majid et al. examined the effect of genistein on the minichromosome maintenance (MCM) gene family. This type of gene is essential for DNA replication and is usually upregulated in various types of cancers, including prostate cancer. In this experiment the researchers observed that treatment of cells with genistein as well as trichostatin A (TSA) significantly downregulated the expression of MCM genes and decreased the S phase of the cell cycle. In addition, there was an observed downregulation of several genes that govern loading of the MCM complex on chromatin. The researchers concluded that treatment of prostate cancer cells with genistein might be advantageous since the MCM genes are an excellent anticancer drug target. In addition, genistein is a natural, non-toxic dietary isoflavone which makes it a very good therapeutic agent, whereas TSA shows high toxicity (94).

A study conducted by Vardi et al. investigated the effects of soy phytoestrogens on the methylation of promoter genes in prostate cancer. This was accomplished using methylation-specific PCR on three different prostate cancer cell lines with the objective to determine the effects of two soy isoflavones (genistein and daidzein) compared to a known demethylating agent as a control. There were two main findings from this experiment: 1) the promoter regions in the prostate cancer cell lines were all strongly methylated in the absence of treatment from the phytoestrogens; and 2) after treatment with phytoestrogens, demethylation of the promoter regions for the tumor suppressor genes (including GSTP1, RASSF1A, EPH2, and BRCA1) occurred and therefore enhanced protein expression was observed. These observations led to the conclusion that modifications to DNA such as the demethylation of the promoter regions in tumor suppressor genes may be related to the protective effect of soy on prostate cancer (95).

An additional study conducted by Adjakly et al. on the demethylating effects of daidzein and genistein produced similar results to the Vardi et al. study. In this experiment, the researchers observed a decrease in methylation percentage in cells treated with genistein and daidzein compared with untreated cells and the corresponding increase in the expression of proteins. These effects were seen on BRCA1, GSTP1, and EPHB2 genes but not on BRCA2 genes. These authors concluded as well that soy phytoestrogens have a protective effect against prostate cancer, but more studies are needed to understand the mechanisms (96).

A study conducted by Phillip et al. investigated the effects of combining genistein and a histone deacetylase inhibitor as treatments against prostate cancer cell survival. Using a whole genome methylation profile, the authors observed the genome wide differences in genetic and epigenetic responses to genistein in prostate cancer cells, and determined the amount of cell proliferation and cell death using the combination treatment. At decreased concentrations of genistein, there was no significant effect on methylation, however the combination of genistein with the histone deacetylase inhibitor showed increased cell death and decreased proliferation. This led to the conclusion that there are a number of pathways that are affected by genistein and there is a synergistic effect with the combination treatment to induce cell death. These findings could be an additional treatment option for early stage prostate cancer (97).

Finally, Chiyomaru et al. investigated the effects of genistein on the regulation of oncogenic microRNA-151 (miR-151) and its target genes. Oncogenic miR-151 is involved in the progression and metastasis of prostate cancer by targeting genes that have been suggested to have a tumor suppressor functionality (CASZ1, IL1RAPL1, SOX17,

NSBP1, and ARHGDIA). Treatment of prostate cancer cell lines with a high expression of miR-151 with genistein showed a significant downregulation of the expression of miR-151 compared with the control and significantly inhibited cell migration and invasion. Further tests showed that the expression levels of the five target genes were significantly different when treated with genistein. These observations led to the conclusion that genistein mediated suppression of oncogenic miRNAs and that genistein ingestion can be an important dietary therapeutic strategy for the treatment of prostate cancer (98).

Clinical Trials

Evidence from the in vitro studies and animal models demonstrates the efficacy of the use of soy products and genistein as a prevention method or treatment option for prostate cancer. In addition to these types of studies, there have been a few relevant clinical trials that investigated the use of soy products in prostate cancer patients in a controlled setting.

A study conducted by Tanaka et al. investigated the association between soy isoflavone supplements and serum levels of sex hormones implicated in prostate cancer development among healthy men. For this trial, a total of 28 healthy Japanese volunteers between the ages of 30 and 59 were given soy isoflavone supplements (60 mg daily) for three months. Of these 28 men, 18 were equol producers and 10 were equol non-producers. Equol is metabolized from the intake of daidzein and exhibits weak phytoestrogen activity. It shows an increased affinity for the estrogen receptor β as well as to the sex hormone-binding globulin (SHBG) and may inhibit the growth of prostate cancer cells. It has been observed in previous studies that only some people are able to metabolize daidzein into equol and the proportions of equol produced differ greatly

among races and age groups. In this experiment the sex hormone levels of the men in the study were measured at baseline and after administration of the soy isoflavone supplements. Additional measurements included the serum and urine concentrations of daidzein and genistein, and the levels of equol in the fasting blood samples. The main results of this study showed: 1) no change in the serum levels of estradiol and total testosterone after the three month supplementation; 2) the serum levels of SHBG significantly increased, and the serum levels of free testosterone and dihydrotestosterone (DHT) decreased significantly after the three month supplementation; and 3) among the 10 equol non-producers, equol became detectable in the serum of two of the healthy volunteers after three month supplementation. These results showed that short-term supplementation with soy isoflavone supplements stimulated the production of serum equol and decreased the serum DHT in healthy volunteers. This suggests the possibility of converting equol non-producers into producers and that a diet based on soy isoflavones may be useful in the prevention of prostate cancer (99).

A true randomized, placebo-controlled, double-blind clinical trial conducted by Lazarevic et al. examined the efficacy and safety of genistein intervention in patients with localized prostate cancer prior to a radical prostatectomy. The trial consisted of 54 study subjects that were randomized to the treatment group (23 subjects) or the placebo group (24 subjects) for three to six weeks prior to radical prostatectomy. The treatment group was given 30mg of synthetic genistein daily in the form of a capsule that was identical in both capsule form and container to the placebo group. Primary outcomes in this trial included PSA levels in the serum and prostatic tissue and the serum testosterone levels. Secondary outcomes in this trial consisted of: 1) pathological investigation of the

removed prostate to analyze changes in the Gleason score, volume of the prostate, and extent of tumor stage compared to preoperative biopsies; 2) safety through the reporting of adverse events; 3) compliance to the study design; and 4) total genistein plasma concentration. The main results of this study included: 1) a reduction of PSA levels by 7.8% in the treatment group and a 4.4% increase in the placebo group ($p = 0.051$); 2) the PSA level in the treatment group was reduced in the tumor tissue; 3) total cholesterol was significantly lower in the treatment group; 4) no significant effects were seen on thyroid or sex hormones; 5) plasma concentrations of genistein were 100 times higher in the treatment group after receiving the treatment ($p < 0.001$; and 6) there were very few adverse events reported and all were mild. These results led to the conclusion that dietary genistein from soy products may reduce serum PSA levels without any effects on hormones and normalized PSA expression in the malignant prostate cancer tissue, meaning there is a possible therapeutic effect by genistein in early prostate cancer (100).

Epidemiologic Studies

There have been a small number of epidemiological studies investigating the association between phytoestrogen intake and the risk of prostate cancer. The objective of these studies was to evaluate the effects of phytoestrogens on prostate cancer risk, through both intake and excretion measurements, on very specific, often small, groups of subjects. None of the epidemiologic studies to this point have evaluated the risk of prostate cancer associated with phytoestrogens in a large US population. In addition, there have been no studies conducted in the US using the NHANES or PLCO data, or utilizing a cohort study design to examine the relationship. Results of the epidemiologic

studies to this point have yielded mixed results. Research conducted in this dissertation will investigate the association between phytoestrogens and prostate cancer in a much larger population in the US, which will add to the generalizability. Additionally, the association with phytoestrogens will be observed separately for advanced and non-advanced prostate cancer, to determine if the risks are different for developing either of these two outcomes.

The earliest studies of the association between phytoestrogen intake or urinary excretion and the resulting risk of prostate cancer showed little to no association. A study conducted by Ganry published in 2006 reviewed previous epidemiologic studies that provided data on 1) dietary soy intake or flavonoid intake; 2) urinary excretion of isoflavones or lignans; or 3) blood measurements of isoflavones or lignans using soy as a marker for isoflavone intake. This review identified eight studies of interest, four case-control studies and four prospective studies. The results of most of these studies showed no association between phytoestrogen intake or urinary excretion and the risk of prostate cancer. One case-control and one prospective study did observe a statistically significant reduced risk. Many of these studies exhibited design limitations and flaws including very small groups of cases and controls, misclassification of soy intake due to measurement errors associated with a dietary instrument, a lack of data on phytoestrogen content in food at the time the study was conducted, a lack of information about portion size of soy product consumption, limiting the study subjects to the Asian population where soy consumption is homogeneously high, and recall bias from the food frequency questionnaire. This study concluded that there appeared to be no association with the intake or urinary output of phytoestrogens and the risk of prostate cancer, however with

the design limitations and flaws make these results difficult to assess and further study is needed (101).

Recent published studies often observed a protective effect between the intake of phytoestrogens and the risk of prostate cancer. A study conducted by Hedelin et al. examined the associations between dietary phytoestrogens and serum enterolactone and the risk of prostate cancer in a Swedish population. In this study, dietary intake of phytoestrogens was assessed using a food frequency questionnaire, and the reported dietary intake of lignans was validated through the measurement of enterolactone in the serum. The association between phytoestrogens and prostate cancer was determined through the reported dietary intake of total and individual phytoestrogens and the serum enterolactone concentration. The study design was a population based case-control study in Sweden with 1,499 prostate cancer cases and 1,130 controls (with serum enterolactone levels available in a smaller subgroup of 209 cases and 214 controls). Results from this case-control study included: 1) high intake of foods rich in phytoestrogens was associated with a significant decrease in the risk of prostate cancer (OR for quartile 4 vs. quartile 1: 0.74; 95% CI: 0.57, 0.95); 2) no observed association between individual phytoestrogens and the risk of prostate cancer; and 3) intermediate levels of enterolactone in the serum was associated with a decreased risk of prostate cancer (OR for quartile 2 vs. quartile 1: 0.28; 95% CI: 0.15, 0.55). From these observations the authors concluded that intake of foods high in phytoestrogens is associated with a decreased risk of prostate cancer, which represents an opportunity for prostate cancer primary prevention (102).

A study conducted by Heald et al. investigated the link between phytoestrogens and the risk of prostate cancer in Scottish men. This study was based on the PCANDIET

study, a population-based case-control study of prostate cancer in relation to inherited susceptibility and diet from the lowlands and central belt of Scotland. Study subjects included 433 cases and 485 controls aged 50-74 years. The levels of phytoestrogen intake were assessed using a self-reported food frequency questionnaire with food values of genistein and daidzein extracted. In addition, a serum concentration of enterolactone was obtained from each subject. Results from this study included: 1) a significant inverse association on prostate cancer risk with increased enterolactone concentrations in the serum (OR: 0.40; 95% CI: 0.22, 0.71); 2) significant inverse association on prostate cancer risk with the consumption of soy foods (OR: 0.52; 95% CI: 0.30, 0.91); and 3) no significant associations observed for dietary intake or serum concentrations of individual phytoestrogens. These results led to the conclusion that the intake of soy foods and enterolactone metabolized from dietary lignans protect against prostate cancer in older Scottish men (103).

A study conducted by Jackson et al. examined the association between the urinary excretion of phytoestrogens and the risk of prostate cancer in Jamaican men. This was a hospital-based case-control study with 175 newly diagnosed cases of prostate cancer and 194 controls from urology clinics of two major hospitals in Jamaica. Men with advanced cases of metastatic cancer, previous prostate surgery, recent severe weight loss, currently on hormonal treatment, or taking finasteride were excluded from the study. Urine samples from these men were tested for concentrations of genistein, daidzein, equol, and enterolactone by time-resolved fluoroimmunoassay. These men were divided into two groups of equol producers and non-producers (similar to other studies), with producers defined as men with detectable urinary concentrations of equol. The results from this

study included: 1) no significant differences in median concentrations of isoflavones from phytoestrogens; 2) compared to non-producers of equol, men who produced equol were at decreased risk of total prostate cancer (OR for tertile 2 vs. tertile 1: 0.42; 95% CI: 0.23, 0.75; OR for tertile 3 vs. tertile 1: 0.48; 95% CI: 0.26, 0.87); 3) compared to non-producers of equol, men who produced equol were at decreased risk of advanced prostate cancer (OR for tertile 2 vs. tertile 1: 0.31; 95% CI: 0.15, 0.61; OR for tertile 3 vs. tertile 1: 0.29; 95% CI: 0.13, 0.60); 4) higher concentrations of enterolactone were positively associated with an increased risk of total prostate cancer (OR: 1.85; 95% CI: 1.01, 3.44); 5) higher concentrations of enterolactone were positively associated with increased risk of advanced prostate cancer (OR: 2.46; 95% CI: 1.11, 5.46); and 6) there were no associations between urinary excretion of genistein and daidzein and the risk of prostate cancer. These results led to the conclusion that equol producers may be at reduced risk of total and advanced prostate cancer, however enterolactone may increase the risk of total and advanced prostate cancer (104).

A study conducted by Park et al. examined the association between urinary phytoestrogen excretion and prostate cancer risk in a group of men aged 45 – 75 years. The study design was a nested case-control within a large multiethnic cohort in Hawaii and California. The Multiethnic Cohort Study was established in Hawaii and the Los Angeles area of California from 1993-1996 and contained over 215,000 adults who completed a detailed questionnaire on diet and lifestyle factors and came from five ethic/racial groups: African American, Native Hawaiian, Japanese American, Latino, and White. A smaller subcohort from the larger cohort study had biological specimens of blood and/or urine collected, largely between 2001 and 2006. A total of 249 cases and

404 matched controls were determined to be eligible for the nested case-control study. Levels of daidzein, genistein, equol, and enterolactone were analyzed in the urine by HPLC to determine concentrations. Results from this study included: 1) an inverse association with total prostate cancer risk observed for daidzein (OR for quintile 5 vs. quintile 1: 0.55; 95% CI: 0.31, 0.98) across all ethnic groups; and 2) no significant association was detected for the risk of total prostate cancer and excretion of genistein, equol, or enterolactone. These results led to the conclusion that high intake of isoflavones reflected by the excretion in the urine of daidzein may be responsible for a protective effect against prostate cancer (105).

There were two separate studies conducted by Travis et al. based on the European Prospective Investigation into Cancer and Nutrition Cohort (EPIC). The first study, published in 2009, examined the association of plasma concentrations of phytoestrogens in relation to the risk of prostate cancer using a nested-case control design of the EPIC cohort study. This study identified 950 prostate cancer cases and 1,042 matched controls from the eight participating countries, Denmark, Germany, Greece, Italy, the Netherlands, Spain, Sweden, and the United Kingdom. Cases were defined as men who developed prostate cancer after the period of blood collection in the study but before the end of the study period. Exclusions included cases with no blood collection information or those who had a history of another type of cancer other than melanoma. A smaller number of participants (675) had information available on tumor stage, of which 475 were classified as localized prostate cancer and 200 were classified as advanced stage prostate cancer. Matching criteria for the controls included recruitment center, age at enrollment within six months, time of day of blood collection within one hour, follow-up

time, and time between blood draw and the last consumption of food or drink. Plasma samples for cases and controls were analyzed for three isoflavones (genistein, daidzein, and equol) and two lignans (enterolactone and enterodiol). Relative risks for prostate cancer in relation to plasma concentrations of the three phytoestrogens were estimated by conditional logistic regression. The results of this study included: 1) higher concentrations of genistein were associated with a lower risk of prostate cancer (RR for quintile 5 vs. quintile 1: 0.74; 95% CI: 0.53, 0.96) and after adjustment for potential confounders the results were borderline significant (RR for quintile 5 vs. quintile 1: 0.74; 95% CI: 0.54, 1.00); and 2) there were no statistically significant associations for the risk of prostate cancer observed for circulating concentrations of daidzein, equol, enterolactone, or enterodiol. These results led to the conclusion that higher concentrations of circulating genistein may reduce the overall risk of prostate cancer, but the evidence from this study does not support an association between plasma lignans and prostate cancer (106). The second study conducted by Travis et al. was published in 2012 as an extension of the first study. This study added an additional 655 cases and 655 matched controls using their original inclusion and exclusion criteria for a total of 1,605 cases and 1,697 matched controls. The results from this larger follow-up study contradicted the results from the first study, showing no observed association between genistein concentration and a decreased risk of prostate cancer. The contradictory nature of these findings may be explained from only measuring the serum concentrations of circulating genistein once instead of multiple times to obtain an average. In a population with a relatively low intake of isoflavones, one measurement may not be a reliable indicator of long-term exposure to genistein and other isoflavones associated with soy

products. The authors also stated that measurement error may be masking an association that exists between genistein and prostate cancer risk (107).

A study conducted by Ward et al. was based on the European Prospective into Cancer and Nutrition (EPIC) Norfolk data, with subjects ranging from between the ages of 45-75. One objective of this study was to investigate the association between phytoestrogen concentrations measured in the serum and urine and the risk of prostate cancer. From the larger cohort study, the authors used a nested case-control design, and identified 193 incident cases of prostate cancer with 828 controls. The prostate cancer cases were identified a minimum of 12 months after enrollment into the EPIC-Norfolk study. Data collected in this study included health and lifestyle variables, a 7-day food diary, anthropometric measurements, and laboratory measures that included concentrations of phytoestrogens. In this study the results of a logistic regression analysis, after the adjustment for age, height, weight, and intake of energy, fat, and lycopene, included: 1) no significant association between prostate cancer risk and total urinary isoflavones (OR: 1.01; 95% CI: 0.93, 1.10) or total urinary lignans (OR: 0.94; 95% CI: 0.86, 1.04); and 2) similar null associations observed for all urinary and serum levels of individual phytoestrogen biomarkers. These results led to the conclusion that there was no evidence to support an association between phytoestrogen exposure and a reduced risk of prostate cancer (108).

Another study by Ward et al., published in 2010, used updated data from the same database and phytoestrogen intake from a 7-day dietary instead of serum and urinary concentrations of individual phytoestrogens used in the previous study. This study identified 204 cases of prostate cancer and 812 controls. The results of the multivariate

analysis (controlling for age, height, weight, physical activity, social class, family history of prostate cancer, and daily intake of energy, fat, zinc, selenium, and lycopene) included: 1) no observed association between the intake of total phytoestrogens and the risk of prostate cancer (OR: 0.86, 95% CI: 0.66, 1.13); and 2) there was a positive association approaching significance between the intake of equol and the risk of prostate cancer (OR: 1.31, 95% CI: 1.00-1.71). From these results the authors concluded that there was no association between the intake of phytoestrogens and the risk of prostate cancer, but equol may influence the risk of prostate cancer (109).

Phytoestrogens and Cardiovascular Disease

Biologic Effects

Studies on the biological effects of phytoestrogens and cardiovascular disease, specifically animal models, in-vivo/in-vitro studies, and gene studies are scarce.

Animal Models

Only one experimental study based on an animal model was relevant to this research. That study, conducted by Kelly et al., investigated the effects of genistein on gene expression related to cardiovascular disease in rats that were induced to menopause. In this study, fifty-nine Sprague-Dawley rats had both ovaries removed to induce menopause. After this procedure, they were randomized into four treatment groups and one control group. The control group was fed soy-free food, two of the treatment groups were fed soy-free food supplemented with two concentrations of estradiol (0.19 mg and 0.75 mg), and two treatment groups were fed soy-free food supplemented with two

concentrations of genistein (6 mg and 60 mg). After three months, levels of prothrombin, coagulation factor VII, fibrinogen alpha, fibrinogen beta, and C-reactive protein (CRP) (all of which have been linked to cardiovascular disease) were tested in all groups. The researchers discovered that gene expression of prothrombin, factor VII, fibrinogen alpha, and fibrinogen beta were significantly increased in all of the treatment groups compared to the control group. Additionally, the genistein treatment group also increased factor VII significantly more than the estradiol treatment group. Finally, CRP levels were also statistically significantly increased in the treatment groups compared with the control group. Although relating animal models to humans is sometimes difficult, these results suggested that genistein may have an adverse effect on cardiovascular health (110).

In-Vitro Studies

Similar to the experimental study conducted on an animal model, only one in-vitro study showed any relevance to the current research. That study, conducted by Kayisli et al., hypothesized that the isoflavone genistein may be involved in the prevention of coronary heart disease through the mechanism of regulating the survival of human coronary artery endothelial cells (HCAEC). In order to investigate this hypothesis, the researchers performed immunocytochemistry, cell proliferation assay, and apoptosis assay on HCAEC's that were treated with genistein. HCAEC's were obtained from three healthy postmenopausal women with no known history of cardiovascular disease and treated with various concentrations of genistein (111). Postmenopausal women were chosen for this study because of the increase in coronary heart disease that has been attributed to the loss cardioprotection due to estrogen deficiency (112). Control

HCAEC's were treated with the same medium in the absence of genistein. The main findings of this study included a higher affinity for the estrogen receptor β in the genistein treated HCAEC's, decreased HCAEC proliferation in the genistein treatment group in a concentration dependent manner compared to the control cells, and an increase in the number of apoptotic HCAEC's in a time-dependent manner. The results led to the conclusion that genistein may not only inhibit the growth of human coronary artery endothelial cells, but also may stimulate death of the HCAEC's as well. These findings appear to cause a prevention of angiogenesis and therefore contradict findings from observational studies of the cardioprotective effect of genistein in postmenopausal women (111).

Clinical Trials

The majority of published dietary intervention trials that were conducted on the association between phytoestrogens and cardiovascular disease involved small populations, mainly consisting of postmenopausal women.

Two studies observed the association between the phytoestrogen genistein and cardiovascular disease on postmenopausal women. The first study, conducted by Crisafulli et al., observed the effect of genistein on specific cardiovascular disease risk markers. In this study, 60 healthy postmenopausal women between the ages of 52 and 60 were enrolled in a double-blind, placebo controlled, randomized study for six months. The treatment group received 54 mg of genistein per day, and the control group received a placebo. Cardiovascular risk markers were measured at baseline and after six months, and included glucose, insulin, insulin resistance, osteoprotegrin, fibrinogen, and sex-

hormone-binding globulin (SHBG). The genistein treatment group showed a statistically significant decrease in fasting glucose, fasting insulin, and insulin resistance compared to the placebo group. These results led to the conclusion that genistein treatment may have a beneficial effect on some cardiovascular risk markers, and therefore may potentially reduce cardiovascular disease (113). The second study, conducted by Villa et al., also observed the effect of genistein on cardiovascular disease risk markers. In this study, 50 postmenopausal women were enrolled in a randomized, placebo controlled study for 24 weeks. The treatment group received the same daily dose of genistein (54 mg) while the control group received a placebo. The treatment group was further divided into two categories, normoinsulinemic and hyperinsulinemic. Outcome measures for this study included hormonal and lipid assays, oral glucose tolerance test with glycemic, insulin, and C-peptide evaluation, and indexes of insulin sensitivity and endothelial function. The genistein treatment group showed significant improvement in insulin sensitivity and fasting glucose compared to the placebo group. In the hyperinsulinemic group, the authors observed a significant reduction in fasting insulin and that HDL cholesterol levels were significantly improved. These results led to the conclusion that treatment with genistein significantly influenced some of the risk markers of cardiovascular disease in both normoinsulinemic and hyperinsulinemic patients (114).

There are two published studies by Hall et al. on the association between soy isoflavone enriched foods and biomarkers of cardiovascular disease risk in postmenopausal women. The first of these studies examined the association between soy isoflavones and inflammatory biomarkers (115) and the second examined the association between soy isoflavones and markers of lipid and glucose metabolism (116). Both of

these studies used the same group of 117 postmenopausal women, with the study designed as a double-blind, placebo-controlled crossover dietary intervention trial. Women in the intervention group received an isoflavone-enriched cereal bar with 50 mg of total isoflavones (genistein and daidzein) for eight weeks, with an eight-week washout period between the crossover. In the study of isoflavones and inflammatory biomarkers, outcomes of interest included von Willebrand factor, intracellular adhesion molecule 1, vascular cell adhesion molecule 1, E-selectin, monocyte chemoattractant protein 1, C-reactive protein (CRP), and endothelin 1 concentrations. Differences with respect to several single-nucleotide polymorphisms were also investigated. In this study the authors observed an improvement in the intervention group for CRP concentrations, but no other statistically significant changes were observed for the other outcomes of interest. This led to the conclusion that isoflavones have beneficial effects on CRP concentrations in postmenopausal women, but not on other known inflammatory biomarkers (115). In the study of isoflavones and markers for lipid and glucose metabolism, outcomes of interest included total, HDL, and LDL cholesterol, triacylglycerols, lipoprotein A, glucose, nonesterified fatty acids, insulin, and the homeostasis model assessment of insulin resistance. Differences with respect to single-nucleotide polymorphisms were also investigated. In this study the authors observed no significant beneficial effect on plasma concentrations of lipids, glucose, or insulin in the treatment group. They did however observe an increase in HDL cholesterol in the treatment group for one particular genotype of an estrogen receptor β gene. This led to the conclusion that isoflavone supplementation does not affect lipid or other metabolic markers of cardiovascular

disease in postmenopausal women, but women with a particular genotype in an estrogen receptor gene may see an increase in HDL cholesterol (116).

The final dietary intervention trial conducted on postmenopausal women also included healthy men of a similar age. In this study, conducted by Teede et al., the objective was to observe the cardiovascular effects of dietary phytoestrogens delivered in soy products. A group of 108 men and 105 postmenopausal women between the ages of 50-75 participated in this 3-month, double-blind, placebo controlled, randomized dietary intervention trial. Individuals in the intervention group received a soy protein isolate with 40 g of soy protein and 118 mg of isoflavones, while individuals in the control group received a placebo. Outcomes of interest in this study included blood pressure, lipids, vascular function, and endothelial function. In the intervention group, the authors observed a significant decrease in blood pressure, a reduction in the LDL to HDL ratio, a reduction in triglycerides, and an overall improved arterial function. Adverse effects observed included a decline in endothelial function in males and an increase in lipoprotein A. This combination of observed results led to the conclusion that further research is need in the subpopulations of hypertensive and hyperlipidemic individuals, in order to discover a more definitive association (117).

Another randomized, double-blind, placebo controlled trial was conducted by Qin et al. on hypercholesterolemic adults aged 40-65 years. In this study, the objective was to evaluate the effects of the isoflavone daidzein on cardiovascular disease risk factors in adults with high cholesterol. The 177 participants that completed this study were randomized to one of three groups, the placebo (control) group, and two intervention groups, the first receiving 40 mg of daidzein daily, and the second receiving 80 mg of

daidzein daily, for six months. Outcomes of interest for this study included fasting lipid profiles, glucose, serum uric acid concentrations, serum lipoprotein A concentrations, blood glycated hemoglobin, and serum insulin. The results of this study included a significant decrease in both treatment groups of serum triglycerides and serum uric acid compared to the placebo group, but no additional significant differences were observed for any of the other outcomes of interest. Greater decreases in serum triglycerides were observed for the GA genotype of the estrogen receptor β gene RsaI. These results led to the conclusion that daidzein consumption may improve certain cardiovascular risk factors, especially in adults with the GA genotype of the estrogen receptor β gene RsaI (118).

Finally, a study was conducted by Wiseman et al. examining the association between soy protein containing isoflavones and biomarkers of lipid peroxidation and resistance of LDL oxidation. The design of this study was a randomized, controlled, crossover design of 24 adults between the ages of 19 and 40 years of age. Subjects in this study were given either a soy protein supplement that was high in isoflavones or a soy protein supplement in which most of the isoflavones had been removed through alcohol extraction. Each of the treatment periods lasted for 17 days, with a 25-day washout period between the crossover. Outcomes of interest for this study included plasma concentrations of F2-isoprostane and 8-epi-prostagalndin F2α (biomarkers of lipid peroxidation), and the resistance of LDL to copper induced oxidation. Results from this study included a significant reduction in the plasma concentrations of 8-epi-prostagalndin F2α and an increased lag time for copper-ion induced LDL oxidation in the high isoflavone treatment group. These results led to the conclusion that the consumption of

naturally occurring isoflavones showed an antioxidant effect that may signal a significant reduction in the risk of atherosclerosis, cardiovascular disease, and cancer (119).

Epidemiologic Studies

Similar to the clinical/intervention trials concerning the association between phytoestrogens and cardiovascular disease, many of the epidemiologic studies published on this topic have focused on postmenopausal women. Research conducted in this dissertation for the association between phytoestrogens and cardiovascular disease will utilize a larger population of US adults, aged 18 years and older, which will not only investigate the association in a larger group, but should also increase the generalizability of the results.

A study conducted by de Kleijn et al. utilized data from the Framingham study with the objective to investigate the association between intake of dietary phytoestrogens and metabolic cardiovascular disease risk factors among postmenopausal women. This cross-sectional study consisted of data on 939 postmenopausal women taken from the Framingham cohort. Dietary phytoestrogen levels were determined by a food-frequency questionnaire. Cardiovascular disease risk factors of interest included blood pressure, waist to hip ratio, and plasma lipoprotein levels. Analyses were adjusted for age, BMI, hormone replacement therapy use, smoking status, fiber intake, and dietary potassium intake. The main results of this study when comparing the highest quartile of phytoestrogen intake to the lowest include a decreased waist to hip ratio among women for lignan intake, lower triglyceride levels for isoflavones intake, and a lower cardiovascular risk factor metabolic score for isoflavone intake. These results led to the

conclusion that high intake of phytoestrogens in postmenopausal women favorably altered the cardiovascular risk profile (120).

A large, prospective cohort study was conducted by van der Schouw et al. on Dutch women ages 49-70 years. The objective of this study was to determine if habitual low intake of dietary phytoestrogens was associated with an increase in cardiovascular disease risk. Data on 16,165 women was obtained from the Dutch Prospect-EPIC cohort, with the subjects enrolled for a median of 75 months of follow-up. Phytoestrogen levels were estimated through the use of a dietary intake questionnaire. Cox proportional hazards regression was used to estimate the hazard ratios of cardiovascular disease for quartiles of dietary phytoestrogen intake. Variables that were controlled in the model included age, BMI, smoking status, physical activity, hypertension, hypercholesterolemia, hormone replacement therapy use, menopausal status, and dietary intake of total energy, fiber, fruits and vegetables, and alcohol. The overall results of this study showed no significant association between isoflavones or lignans with cardiovascular disease risk; however, a significant decreased risk of a cardiovascular event was observed for increased lignan intake among smokers. These results led to the conclusion that there is no protective effect associated with a higher intake of phytoestrogens associated with cardiovascular disease (121).

Finally, a study conducted by Pellegrini et al. examined the association between plant lignans and both vascular inflammation and endothelial dysfunction among a group that consisted of postmenopausal women and men of a similar age in Northern Italy. This was a cross-sectional study design consisting of 242 total subjects (151 males and 91 females). The subjects were chosen from a population of workers at a food company in

Italy who had originally completed a survey on risk factors for type II diabetes and cardiovascular disease. Subjects enrolled in this study completed a medical history questionnaire, a three-day food record, and several lab tests to measure the cardiovascular disease outcomes of interest. These outcomes included serum insulin levels, C-reactive protein levels, soluble intercellular adhesion molecule-1, fasting plasma glucose, total cholesterol, HDL cholesterol, triacylglycerol, and brachial flow-mediated dilation. The main results of this study included a significant decreased concentration of soluble intercellular adhesion molecule-1 and a significant increase in brachial flow mediated dilation associated with an increased intake of lignans. These results led to the conclusion that the intake of dietary lignans is associated with a decrease in vascular inflammation and endothelial dysfunction among older adults in Northern Italy (122).

There have been very few epidemiologic studies that have focused on a healthy adult population instead of specific populations such as postmenopausal women. One such study, conducted by Guo et al., examined the relationship between the daidzein metabolites, equol and O-desmethylangolensin and the association with serum lipids and uric acid in an adult population in China. A total of 210 subjects between the ages of 20 and 69 were recruited into this cross-sectional study. Data were collected on fasting serum lipids, glucose, and uric acid, along with both dietary intake and urinary concentrations of phytoestrogens. The main results of this study included a significantly decreased serum uric acid and waist to hip ratio among subjects who produced equol. No significant differences were observed in serum lipids, glucose, and uric acid among subjects who produced O-desmethylangolensin. This led to the conclusion that

individuals who produce equol may show a decreased risk of cardiovascular disease compared to those who are non-producers (123).

Rationale for Research Reported in this Dissertation

Biological evidence exists in the form of both animal models and in vitro studies that suggests that an increased intake of phytoestrogens should produce a protective effect against prostate cancer, however, evidence suggesting a protective association between phytoestrogens and cardiovascular disease has thus far not been adequately researched. Small clinical trials have observed that an increase in phytoestrogen intake may have therapeutic effects towards the prevention and treatment of both prostate cancer and cardiovascular disease. Epidemiologic studies to this point have produced mixed results with some studies showing an inverse relationship between phytoestrogens and prostate cancer or cardiovascular disease risk and others showing no association. Most published epidemiologic studies have focused only on the association in a specific group. In addition, there have been very few epidemiologic studies conducted on the relationship between phytoestrogen intake and the risk of prostate cancer in the United States and even fewer examined the differences in the relationship between phytoestrogen intake and the risk of total versus advanced prostate cancer. To this point there have been no epidemiologic studies on this topic published utilizing the data from the National Health and Nutrition Examination Survey (NHANES) or the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO). Both of these studies contain a great deal of data with the potential to produce results on the relationship between phytoestrogens and prostate cancer or cardiovascular disease among a large population in the US.

Outline of the Dissertation

The research for this dissertation utilized the NHANES and PLCO data to investigate the relationship between phytoestrogen intake and the risk of prostate cancer or cardiovascular disease. Although there are several types of hormone related cancers that could be influenced by the dietary intake of phytoestrogens, this research chose to focus on prostate cancer for several reasons: 1) prostate cancer affects a large population of men in the US (4); 2) most of the established risk factors for prostate cancer are non-modifiable (4, 5), and many modifiable factors have not been adequately explored; and 3) biological evidence suggests that advanced and non-advanced cases of prostate cancer may not be etiologically similar (124), which means that the risk factors may be different as well. In the first study using the NHANES data, the investigation focused on the relationship between urinary excretion of total and individual phytoestrogens and the risk of total cancer, cardiovascular disease, and all-cause mortality. Previous epidemiologic studies focused on the incidence of these diseases and not the mortality. This will be the first time that the NHANES data has been used for this type of study. In the second study, using data from the PLCO, the investigation focused on the relationship between the dietary intake of total and individual phytoestrogens and the risk of the development of total, advanced, and non-advanced prostate cancer. The division of prostate cancer into both advanced and non-advanced cases in the US and the association with phytoestrogens has thus far not been studied. In the third study using NHANES data, the investigation focused on the relationship between urinary excretion of total and individual phytoestrogens and the levels of C-reactive protein (CRP). CRP is a common biomarker for inflammation that has been associated with an increased risk of both cardiovascular

disease and certain types of cancer. The results of these studies using the large NHANES and PLCO datasets should add valuable knowledge on the relationship between phytoestrogens and these chronic conditions.

Chapter 2

Urinary Phytoestrogens and Cancer, Cardiovascular, and All-Cause Mortality in the Continuous National Health and Nutrition Examination Survey

Abstract

Scope: Experimental studies suggest that phytoestrogen intake alters cancer and cardiovascular risk. This study investigated the associations of urinary phytoestrogens with total cancer (n=79), cardiovascular (n=108), and all-cause (n=290) mortality among 5,179 participants in the continuous National Health and Nutrition Examination Survey (1999-2004).

Methods and Results: Survival analysis was performed to evaluate hazard ratios (HRs) and 95% confidence intervals (CIs) for each of the three outcomes in relation to urinary phytoestrogens. After adjustment for confounders, higher urinary concentrations of total lignans were associated with a reduced risk of death from cardiovascular disease (HR for tertile 3 vs. tertile 1: 0.48; 95% CI: 0.24, 0.97), whereas higher urinary concentrations of total isoflavones (HR for tertile 3 vs. tertile 1: 2.14; 95% CI: 1.03, 4.47) and daidzein (HR for tertile 3 vs. tertile 1: 2.05; 95% CI: 1.02, 4.11) were associated with an increased risk. A reduction in all-cause mortality was observed for elevated urinary concentrations of total lignans (HR for tertile 3 vs. tertile 1: 0.65; 95% CI: 0.43, 0.96) and enterolactone (HR for tertile 3 vs. tertile 1: 0.65; 95% CI: 0.44, 0.97).

Conclusion: Some urinary phytoestrogens influenced cardiovascular and all-cause mortality in a representative sample of the US population.

Introduction

Cardiovascular disease and cancer are the leading causes of death in the United States (1) and many other developed countries throughout the world (2). In the United States, 597,689 cardiovascular deaths and 574,743 cancer deaths occurred in 2010 (1). On a global scale, cardiovascular disease was estimated to account for over 13.2 million deaths in 2011 (2), and total cancers claimed an estimated 8.2 million lives in 2012 (3). To prevent the development of cancer and cardiovascular disease, it is necessary to identify their risk factors, particularly modifiable ones. One such modifiable factor is diet.

Phytoestrogens are a group of non-steroidal plant metabolites. The principal classes of phytoestrogens include isoflavones and lignans. Isoflavones abound in soy products, legumes, and chick peas (43, 125), and lignans primarily originate from seed oils, whole grain cereals, and beans (126). Isoflavones found in soy products include genistein, daidzein, and glycitein (127), with these compounds arising after metabolism by the gut bacteria of the glycoside conjugates (128). Daidzein can be further converted into two endogenous metabolites, equol and O-desmethylangolensin, with individual variation in the metabolism of daidzein in populations (129, 130). Plant lignans commonly consumed by humans are converted into mammalian lignans, enterolactone and enterodiol, by the intestinal bacteria (131). Differences in the biochemistry and food sources of individual phytoestrogen compounds requires investigation of total phytoestrogens and their metabolites in relation to disease risk.

A growing body of experimental evidence suggests that it is biologically plausible that phytoestrogen intake may modulate the risk of cancer and cardiovascular disease (132, 133). Phytoestrogens can induce biologic responses due to their structural

similarity to 17β-estradiol when they are consumed in the diet (12). The biologic responses from phytoestrogens include estrogenic, anti-estrogenic, anti-oxidative, anti-viral, anti-bacterial, and anti-proliferative effects (131). It has been found that the potential beneficial effect of phytoestrogens on some hormone-related cancers (134, 135) is mediated through their competitive binding to estrogen receptors (136, 137). While estradiol exhibits an equal affinity to both α and β receptors (ERα and ERβ), phytoestrogens show a stronger affinity to ERβ (138). For example, genistein has an approximately 30-fold greater affinity to the ERβ, and therefore may cause some clinical effects by selectively triggering this particular receptor (138). Administration of phytoestrogens reduced serum testosterone levels in rats, an established risk factor for prostate cancer (132, 139). It was also found that soy phytoestrogens reversed severe pulmonary hypertension and prevented heart failure in the same animals (133).

Despite experimental evidence, few epidemiologic studies have examined the association between phytoestrogen intake and cancer or cardiovascular mortality in Western populations. Previous studies have focused on a few sites of cancer, mainly prostate (140, 141) and breast (142, 143), yielding mixed results. Little is known about the association between phytoestrogen intake and cardiovascular disease (144), although it is considered a promising area of research for cardiovascular disease prevention (145). The consumption of soy products is lower in Western countries than in Asian countries (135, 146). However, several studies have reported a considerable between-person variation in phytoestrogen intake in Western populations (121, 147). This suggests that it is feasible to investigate the effect of phytoestrogens on health and disease in non-Asian countries. Several studies have shown that urinary excretion of phytoestrogens is a

reliable biomarker of phytoestrogen intake (131, 148), as evidenced by a statistically significant correlation between dietary intake of phytoestrogens (particularly from soy products) and their urinary excretion (149). To date, no epidemiologic studies have evaluated the associations between phytoestrogen intake and total cancer, cardiovascular, and all-cause mortality in a national representative sample of the US population. Therefore, the present study investigated this research question using data on urinary excretion of total and individual phytoestrogens as well as total cancer, cardiovascular, and all-cause mortality, previously collected from the continuous National Health and Nutrition Examination Survey (NHANES).

Subjects and Methods

Study Population

Data analyzed in this study were obtained from the NHANES for the years 1999-2004 and the NHANES linked mortality public-use file. The mortality file was created from a follow-up study of mortality that matched records from the individual years of the NHANES study with data in the National Death Index (NDI) through December 31, 2006 (150). These data sources were selected because urinary phytoestrogen data for this six-year period only have been linked to mortality data in the NDI. NHANES is an annual cross-sectional study initiated in 1999 by the Center for Disease Prevention and Control (CDC) to assess the health and nutritional status of the general US population. Data collection and sampling procedures for NHANES have been described in detail elsewhere (151). Sample weights were applied to the data through the calculation of a six-year weight variable according to the guidelines from the National Center for Health

Statistics (NCHS) when combining two or more two-year cycles of the continuous NHANES data to produce an unbiased national estimate.

From 1999 to 2004, a total of 29,402 individuals enrolled in the NHANES also completed the interview and health examination components. As the objective of the present study is to investigate urinary phytoestrogens in relation to cancer, cardiovascular, and all-cause mortality, our analyses were confined to subjects who were ≥18 years and completed the 24-hour dietary recall, reducing the sample size to 17,061. Urinary concentrations of phytoestrogens were measured among approximately one-third of total NHANES participants. Subsampling in NHANES was performed to reduce participant burden and facilitate scheduling and completion of examinations. All subjects in the subsample were randomly selected from the pool of total participants to obtain a nationally representative sample, with subsample weights calculated to account for the probability of being selected into the subsample and additional non-response (152). Excluding subjects without data on urinary phytoestrogens left the cohort with 5,179 subjects, from whom 79 cancer deaths, 108 cardiovascular deaths, and 290 all-cause deaths were identified during a mean follow-up of approximately five years (1999-2006). The de-identified data analyzed in the present study are freely available in public domains, and the approval for such data analysis by the Institutional Review Board of Indiana University was sought but determined not to be applicable.

Baseline Data Collection

NHANES participants were interviewed to collect data on age, sex, race (non-Hispanic white, non-Hispanic black, and other race including multiracial), marital status (married or living with partner, widowed, divorced, or separated, and never married), and education level (less than high school, high school graduate or equivalent, and more than high school). Data were also collected on smoking status [never smokers (smoking 0 or <100 cigarettes in lifetime), former smokers (smoking ≥100 cigarettes in lifetime but not currently smoking), and current smoker], alcohol consumption (0 drink/week, <1 drink/week, and >1 drink/week), and nutrient intake through a 24-hour food recall. Body mass index (BMI) (kg/m^2) was calculated from height and weight measured during the medical examination portion of data collection.

Urinary Phytoestrogen Measurement

Phytoestrogen biomonitoring was accomplished by measuring urinary excretion of isoflavones (including daidzein, genistein, equol, and O-desmethylangolensin) and lignans (including enterodiol, and enterolactone) using high performance liquid chromatography (HPLC) with tandem mass spectrometric (MS/MS) detection (153). The methods for the collection and analysis of urine samples for phytoestrogen concentrations have been described in detail elsewhere (154). Briefly, spot urine specimens were collected at the Mobile Examination Centers the morning after a recommended fast, processed, stored at -20^0C, and then shipped to the Division of Environmental Health Laboratory Sciences at the NCHS for analysis. Urine samples were amended with stable isotope-labeled internal standards to improve method accuracy and precision, incubated

with a deconjugation enzyme to allow the quantification of individual phytoestrogens, extracted using solid phase extraction to remove interferences and improve sensitivity, and then analyzed using negative ion mode electrospray ionization HPLC-MS/MS, an assay with a high degree of specificity for each analyte (154).

Mortality Follow-up

International Classification of Diseases 10th Revision (ICD-10) codes were used in the selected databases that recorded cause-specific deaths ascertained during follow-up through December 31, 2006 (150). The underlying causes of death were grouped according to the guidelines provided by the NCHS. The primary outcomes of the present study were cancer mortality (ICD-10 codes, C0-C97), cardiovascular mortality (ICD-10 codes, I00-I99), and all-cause mortality (155).

Statistical analysis

The study population was divided into tertiles based on individuals' urinary concentrations of both total and each individual phytoestrogen to allow for an adequate number of subjects in each group. Total phytoestrogens were calculated by summing up all of the individual phytoestrogens, with a similar calculation completed for both total isoflavones and total lignans. Demographic, anthropometric, and lifestyle characteristics of subjects (including age, gender, race, BMI, education, smoking status, and alcohol intake) were compared by the tertiles of total urinary phytoestrogens (ng/ml) (tertile 1: 4 – 414; tertile 2: 415 – 1,047; tertile 3: 1,048 – 112,457). Chi-square tests and analysis of variance were employed to compare differences in categorical and continuous variables

among tertiles, respectively. Urinary concentrations of total and individual phytoestrogens were summarized by medians and interquartile ranges. Two-sided t-tests were used to compare them between groups using log-transformed values to account for skewed distributions.

Cox proportional hazards regression was performed to calculate hazard ratios (HRs) and 95% confidence intervals (CIs) for cancer, cardiovascular, and all-cause mortality in relation to urinary phytoestrogens. The lowest tertile of urinary concentration was the reference group to estimate HRs and 95% CIs for two upper tertiles. The variables adjusted in the multivariable models were age, BMI, education, smoking status, total energy intake, sodium intake, and urinary creatinine. No interactions were found to be statistically significant, and thus no interaction terms were included in the final model. Factors that were tested for their interactions with urinary phytoestrogens in relation to each of the three outcomes included age, gender, BMI, education, smoking status, total energy intake, and sodium intake. Gender, race, marital status, and intake of alcohol, fat, and calcium were examined as potential confounders but not included in the final models because they were not statistically significant or did not substantively alter risk estimates (<10%). Two-sided p-values of <0.05 were considered statistically significant. SAS version 9.4 (Cary, NC) was used for all statistical analyses.

Results

Characteristics of study subjects are shown in **Table 1**. Subjects were statistically significantly different across total phytoestrogen tertiles for gender, race, education, smoking status, and alcohol intake. Those in the highest tertile of urinary phytoestrogens

were more likely to be male, non-Hispanic white, have more years of education, and be never smokers, but were less likely to be obese and non-drinkers.

Table 2 shows differences in urinary concentrations of total and individual phytoestrogens between subjects who died of total cancer, cardiovascular disease, and all causes with those who remained alive during follow up through the censor date (December 31, 2006). The median urinary concentrations of total phytoestrogens were lower in cases of death from each of the three outcomes examined than respective non-cases. Similarly, lower urinary concentrations of total lignans were observed for cases of death from cardiovascular disease and all causes, and lower urinary levels of enterolactone were found for cases of death from all causes. Conversely, the median urinary concentrations of total isoflavones and daidzein were higher among subjects who died of cardiovascular disease and all causes than those who remained alive. No significant differences in log-transformed means of total and individual phytoestrogens existed between cases of death and non-cases for each of the three outcomes of interest.

Risk estimates for each of three outcomes examined in relation to urinary excretion of total and individual phytoestrogens are presented in **Tables 3, 4, and 5**. After adjustment for confounders, total phytoestrogens and each individual phytoestrogen were not associated with a significantly altered risk of death from total cancers. A significantly increased risk of death from cardiovascular disease was found for higher urinary excretion of isoflavones (HR for tertile 3 vs. tertile 1: 2.14; 95% CI: 1.03, 4.47) and urinary daidzein (HR for tertile 3 vs. tertile 1: 2.05; 95% CI: 1.02, 4.11). Conversely, higher lignan excretion was significantly associated with a reduced risk of death from cardiovascular disease (HR for tertile 3 vs. tertile 1: 0.48; 95% CI: 0.24, 0.97). Similarly,

a significantly reduced all-cause mortality was found for higher urinary excretion of total lignans (HR for tertile 3 vs. tertile 1: 0.65; 95% CI: 0.43, 0.96) and enterolactone (HR for tertile 3 vs. tertile 1: 0.65; 95% CI: 0.44, 0.97).

All models met the proportional hazard assumption except for the model constructed for urinary enterodiol and cardiovascular mortality. Their association was evaluated by adding an interaction term between enterodiol and the log of time. Consequently, risk for subjects in the second tertile was not statistically significant, but risk for those in the third tertile attained significance level. The addition of the interaction term showed a promoting effect of enterodiol on cardiovascular mortality at a follow-up time of ≤19.9 months, but a protective effect at >19.9 months.

To evaluate the possibility of reverse causality arising from preexisting chronic diseases, additional analyses were performed by removing individuals from the dataset who died within two years of enrollment into the study (156, 157). An increased risk of cancer death was observed for subjects in the second tertile of urinary isoflavones (HR: 2.62; 95% CI: 1.13, 6.10), but risk estimates for all other phytoestrogens remained insignificant. An increased risk of cardiovascular death persisted for subjects in the third tertile of urinary isoflavones (HR: 2.79; 95% CI: 1.10, 7.06), but an increased risk for individuals in the third tertile of urinary daidzein and a decreased risk for those in the third tertile of urinary lignans (HR: 0.39; 95% CI: 0.15, 1.00) were no longer significant. The reduced risk of all-cause mortality disappeared for subjects in the third tertile of urinary lignans and the third tertile of urinary enterolactone.

Discussion

The present study investigated the associations between urinary phytoestrogens and cancer, cardiovascular, and all-cause mortality using data collected from a nationally representative sample of the US population. It was found that urinary concentrations of total lignans were significantly and inversely associated with cardiovascular and all-cause mortality, whereas urinary concentrations of total isoflavones and daidzein were significantly and positively associated with cardiovascular mortality. In addition, higher urinary concentrations of enterolactone was significantly associated with lower all-cause mortality.

Genistein is a main isoflavone present in soy products and has been one of the most widely investigated phytoestrogen metabolites. The present study did not show a significant association between urinary genistein and total cancer mortality, which was consistent with the results of several other studies in which genistein intake was not associated with the risk of different types of cancer (140, 141, 158). Some studies have reported an inverse association between plasma concentrations of genistein and the risk of prostate and breast cancers (159, 160). A few experimental studies revealed a protective effect of genistein on prostate cancer (161, 162), whereas another experimental study reported an increased risk of colon cancer associated with genistein intake (163). In one experiment, Liss et al. found that total phytoestrogens (derived from soy products) were more effective than purified genistein on inhibiting the proliferation of prostate cancer cell lines. In that study, genistein was thought to be the major contributor to the effect of soy products on cancer cells. However, gene expression data suggested that some interaction existed between genistein and other phytoestrogen compounds available

in soy products because genistein alone did not produce the same results (164). Collectively, all the studies discussed above suggest that dietary intake of individual isoflavones or lignans may exert different effects on individual types of cancer. Given the small number of total cancer deaths (n=79) in the present study, it was not possible to examine cancer-specific associations with total and individual phytoestrogens, an intriguing question worthy of investigation in cohort studies with a larger number of cases of common cancers.

Enterolactone is a main lignan metabolite in both urine and blood (131). The concentrations of this metabolite were found to reflect the habitual dietary intake of plant lignans (129). As the precursors of enterolactone are detected in whole-grain products, legumes, seeds, fruits, and vegetables, the urinary concentrations of enterolactone are considered a biomarker for an overall healthy diet (140). The present study showed low all-cause mortality associated with elevated urinary excretion of both total lignans and enterolactone. The consumption of lignan-rich foods has been associated with a decreased risk of breast and prostate cancers in some studies (135) and an increased risk of prostate cancer in other studies (165). The present study did not show a significant association between urinary excretion of total or individual lignans and total cancer mortality. It has been found that enterolactone suppressed the proliferation and migration of prostate cancer cells (166), which suggests that enterolactone intake may reduce the risk of prostate and some other cancers. The differential effects of enterolactone intake on the risk of different sites of cancer (135, 165) may account in part for the null results observed for this compound in relation to total cancer mortality in the present study. A significantly reduced risk of cardiovascular death associated with urinary excretion of

lignans was observed in the present study, which partially contributes to its inverse association with all-cause mortality.

Experimental and epidemiologic data are scarce examining the influence of intake of total and individual phytoestrogens on cardiovascular health and disease. One study showed that a lignan-rich diet was associated with elevated high-density lipoprotein (HDL) concentrations and reduced triglyceride concentrations among US adults (167). As expected, increased serum concentrations of enterolactone have been associated with a reduced risk of acute coronary events and death from cardiovascular disease (168, 169). The results from these previous studies are consistent with those of the present study. Additionally, the present study showed an increased risk of cardiovascular death associated with urinary excretion of total isoflavones and daidzein. The results of previous studies on these associations are conflicting. A placebo-controlled, double-blinded trial of postmenopausal women supplemented with isoflavone soy protein showed no statistically significant effect on atherosclerosis progression (170). Similarly, a meta-analysis of randomized controlled trials revealed that isoflavone supplementation did not improve endothelial function in postmenopausal women with high baseline flow-mediated dilation levels, but significant benefits were found for those with low baseline flow-mediated levels (171). A cross-sectional study on middle-aged men in the US reported that usual intakes of isoflavones were not associated with a favorable cardiovascular risk profile (172). A protective or null effect of isoflavones on cardiovascular disease that was observed in previous studies was inconsistent with a deleterious effect that was found in the present study. This difference might arise from two reasons: 1) most previous studies have focused on postmenopausal women; 2) in

those studies, indicators of cardiovascular functions or biomarkers of cardiovascular lesions were examined; instead, the present study evaluated urinary excretion of total isoflavones and daidzein in relation to cardiovascular mortality among adult women and men of all ages.

The present study has several advantages. Exposure to total and individual phytoestrogens was evaluated by measuring their concentrations in spot urine. Urinary excretion of phytoestrogens is free of recall bias inherent in food frequency questionnaires and is an integrated reflection of phytoestrogen intakes from all sources, including those that may be inadequately represented in food composition databases. For example, the most abundant sources of isoflavones in the diet are from foods containing soy products, such as tofu. However, soy additives are found in some processed foods (173), and certain isoflavones are naturally present in lower concentrations in other foods such as vegetables (174), fruits, and nuts (175). Another theoretical advantage of measuring urinary phytoestrogens is that this assay can also capture phytoestrogen metabolites (e.g. equol and O-desmethylangolensin) produced by intestinal bacteria (71). It is critical to determine amounts of exposure to specific phytoestrogens because they differ in their levels of biological activity (135). Most food composition databases have insufficient data on individual phytoestrogens and their metabolites, which make it difficult to reliably quantify intake of these bioactive compounds using food frequency questionnaires. Most previous investigations of the effect of phytoestrogens on cancer risk were small case-control studies (165, 176). Another strength of the present study is that the analysis prospectively evaluated association between urinary phytoestrogens and all-cause and cause-specific mortality. The data used are based on a nationally

representative sample with a relatively large between-person variation in urinary excretion of individual and total phytoestrogens.

Limitations of the present study need to be considered in the interpretation of obtained results. A small number of events for both cancer mortality and cardiovascular mortality did not allow us to perform a stratified analysis by type of cancer or cardiovascular disease. Future studies that incorporate a longer follow-up period may provide new insights into the etiology of cancers and cardiovascular diseases. Spot urine was used to determine phytoestrogen concentrations, and the results of these measurements might be different from those using 24-hour urine due to potential circadian rhythm. To adjust for variation in urine dilution, the phytoestrogen concentrations were normalized to urinary creatinine, a commonly used method (148, 177), because creatinine is excreted by glomerular filtration at a relatively constant rate (178). There have been no studies examining the correlation between spot and 24-hour urinary phytoestrogen concentrations. However, phytoestrogen concentrations, particularly individual isoflavones, in spot urine have been reported to be statistically significantly correlated with their concentrations measured in serum (179). In addition, urinary biomarkers of phytoestrogens were measured only once, and a single measurement might not accurately reflect individuals' usual dietary intake due to within-person variation. To capture habitual intake of phytoestrogens, repeated measurements of urinary excretion of this family of chemicals may be necessary, but data on such repeated measurements are not available from NHANES due to feasibility limitations.

As data on dietary intake of phytoestrogens were not available from NHANES, it was not possible to determine the correlation between urinary phytoestrogens and their

dietary intake. Significant associations of urinary excretion of daidzein and total lignans with cardiovascular and/or all-cause mortality disappeared after excluding cases of death that occurred within two years of enrollment, which suggests that these associations reported in Tables 4 and 5 may be partially ascribed to reverse causality. Mortality data were analyzed in the present study. Therefore, obtained results may be less relevant to the etiology of total cancer and cardiovascular diseases than those from analysis of incidence data. This is because mortality of these two outcomes may be influenced by differences in access to and quality of medical treatment among study subjects.

It is possible that the effect of phytoestrogens on cancer and cardiovascular disease risk differs by metabolic phenotype. Equol is a metabolite of daidzein and is considered to be the most biologically active phytoestrogen among soybean-derived isoflavones (180). It has been found that 30% to 50% of humans are capable of producing this metabolite (129), although there is still no consensus on the definition of the producers of this compound (129, 181). Previous studies have yielded mixed results on the association between equol-producing status (measured in plasma) and prostate cancer risk. A significantly reduced risk of total prostate cancer was observed among individuals who produced high amounts of equol (158, 159), but this finding was not confirmed in other studies (176, 182). It was not possible to evaluate the risk of death from total cancer and cardiovascular disease by equol-metabolizing phenotypes due to relatively small sample size in the present study.

In summary, the present study suggests that higher urinary concentrations of total lignans were associated with a reduced risk of death from cardiovascular disease. Similarly, elevated urinary concentrations of both total lignans and enterolactone were

associated with low all-cause mortality. Conversely, higher urinary concentrations of total isoflavones and daidzein were significantly associated with an increased risk of death from cardiovascular disease. It is important and timely to further investigate the associations of phytoestrogen intake, its biomarkers, and metabolic polymorphisms with the risk of total cancer, specific cancers, and cardiovascular disease in large prospective cohort studies, as data generated from such studies may offer innovative avenues for the prevention of these major diseases among people across the world.

Table 1: Baseline characteristics of subjects by tertiles of urinary excretion of total phytoestrogens (ng/mL) in the continuous National Health and Nutrition Examination Survey, 1999-2004

	Total Phytoestrogens (ng/mL)			
Characteristics	Tertile 1 (4 - 414) n = 1,726	Tertile 2 (415 - 1,047) n = 1,727	Tertile 3 (1,048 - 112,457) n = 1,726	p-value
Age [Mean (SD)]	44.7 (16.8)	45.5 (17.9)	44.8 (17.4)	0.28
Gender (%)				
Male	45.9	47.2	51.0	0.006
Female	54.1	52.8	49.0	
Race/Ethnicity (%)				
Non-Hispanic White	70.7	71.2	72.5	0.029
Non-Hispanic Black	9.9	11.8	11.7	
Other	19.4	17.0	15.8	
BMI[1] [Mean (SD)]	28.3 (6.4)	28.2 (5.9)	27.7 (6.4)	0.004
Education (%)				
Less than High School	23.1	20.7	19.5	<0.001
High School Graduate or Equivalent	27.3	27.5	22.9	
More than High School	49.6	51.8	57.6	
Smoking Status (%)				
Never Smoker	48.2	51.4	53.6	<0.001
Former Smoker	22.9	24.1	25.3	
Current Smoker	28.9	24.5	21.1	
Alcohol Intake (%)				
0 drinks/week	20.2	21.0	16.7	0.025
< 1 drinks/week	41.9	42.9	46.1	
> 1 drinks/week	37.9	36.2	37.3	

[1] Body Mass Index.

Table 2: Differences in urinary concentrations of total and individual phytoestrogens (ng/mL) between subjects who did and did not die from total cancer, cardiovascular disease, or all-causes in the continuous National Health and Nutrition Examination Survey, 1999-2004[1]

	Total Cancer		Cardiovascular Diseases		All Causes	
	Cases of Deaths	Non-Cases	Cases of Deaths	Non-Cases	Cases of Deaths	Non-Cases
Phytoestrogens	(n = 79)	(n = 5,100)	(n = 108)	(n = 5,071)	(n = 290)	(n = 4,889)
Total Phytoestrogens	607 (416, 1311)	679 (306, 1440)	437 (268, 1083)	682 (308, 1442)	531 (294, 1117)	687 (308, 1453)
Isoflavones	160 (67, 294)	114 (44, 345)	163 (62, 260)	114 (44, 346)	139 (54, 286)	113 (44, 346)
Genistein	32 (13, 88)	26 (9, 89)	28 (13, 79)	26 (9, 90)	31 (12, 79)	26 (9, 90)
Daidzein	78 (28, 170)	56 (18, 191)	84 (32, 143)	56 (18, 191)	68 (21, 167)	56 (18, 191)
Equol	8 (3, 19)	8 (2, 17)	6 (3, 14)	8 (2, 17)	7 (3, 18)	8 (2, 17)
O-desmethylangolensin	3 (0, 16)	4 (1, 19)	5 (1, 21)	4 (1, 19)	3 (1, 16)	4 (1, 19)
Lignans	437 (213, 809)	415 (148, 928)	299 (124, 706)	416 (149, 931)	347 (152, 750)	417 (148, 940)
Enterodiol	53 (18, 112)	39 (14, 92)	32 (16, 66)	40 (14, 93)	33 (15, 86)	40 (14, 93)
Enterolactone	371 (171, 743)	347 (104, 821)	240 (75, 622)	349 (105, 824)	289 (124, 628)	351 (104, 825)

[1] Values are medians (interquartile ranges).

Table 3: HRs (95% CIs) for total cancer mortality by tertiles of urinary concentrations of total and individual phytoestrogens in the continuous National Health and Nutrition Examination Survey, 1999-2004

	Cancer Mortality			
Phytoestrogens (ng/mL)	**No. of Cases**	**Person-Years**	**Creatinine-Adjusted HR (95% CI)[1]**	**Multivariable-Adjusted HR (95 % CI)[2]**
Total Phytoestrogens				
T1 (4-14)	25	1,820	Reference	Reference
T2 (415-1,047)	27	1,906	1.76 (0.93, 3.35)	1.41 (0.72, 2.75)
T3 (1,048-112,457)	27	1,823	1.36 (0.68, 2.71)	1.18 (0.57, 2.46)
p-trend			0.73	0.90
Isoflavones				
T1 (1-58)	20	1,451	Reference	Reference
T2 (59-219)	30	2,081	1.96 (1.00, 3.87)	1.94 (0.96, 3.95)
T3 (220-55,729)	29	2,017	1.62 (0.80, 3.30)	1.67 (0.79, 3.52)
p-trend			0.61	0.56
Genistein				
T1 (0-13)	22	1,606	Reference	Reference
T2 (14-54)	25	1,765	1.57 (0.81, 3.06)	1.44 (0.73, 2.87)
T3 (55-25,700)	32	2,178	1.70 (0.88, 3.31)	1.46 (0.73, 2.93)
p-trend			0.23	0.51
Daidzein				
T1 (0-25)	20	1,441	Reference	Reference
T2 (26-115)	29	2,098	1.41 (0.72, 2.78)	1.29 (0.64, 2.63)
T3 (116-29,200)	30	2,010	1.68 (0.86, 3.29)	1.77 (0.90, 3.49)
p-trend			0.18	0.11
Equol				
T1 (0-3)	22	1,532	Reference	Reference
T2 (4-11)	24	1,722	0.96 (0.48, 1.92)	0.94 (0.46, 1.91)
T3 (12-17,200)	27	1,927	1.12 (0.58, 2.19)	1.12 (0.55, 2.27)
p-trend			0.67	0.69
O-desmethylangolensin				
T1 (0-1)	29	2,124	Reference	Reference
T2 (2-9)	25	1,730	0.91 (0.49, 1.71)	0.78 (0.41, 1.48)
T3 (10-9,890)	23	1,569	0.83 (0.44, 1.56)	0.75 (0.38, 1.48)
p-trend			0.59	0.58
Lignans				
T1 (0-225)	27	1,999	Reference	Reference
T2 (226-691)	30	2,116	1.68 (0.90, 3.13)	1.43 (0.75, 2.73)
T3 (692-85,847)	22	1,434	1.22 (0.62, 2.39)	1.05 (0.52. 2.14)
p-trend			0.87	0.86
Enterodiol				
T1 (0-20)	27	1,991	Reference	Reference
T2 (21-63)	22	1,586	0.94 (0.47, 1.88)	1.09 (0.54, 2.22)
T3 (64-18,000)	28	1,835	1.60 (0.85, 3.01)	1.66 (0.85, 3.34)
p-trend			0.08	0.10
Enterolactone				
T1 (0-173)	25	1,790	Reference	Reference
T2 (174-595)	32	2,330	1.77 (0.96, 3.29)	1.52 (0.80, 2.90)
T3 (596-85,300)	22	1,429	1.19 (0.60, 2.32)	1.01 (0.50, 2.05)
p-trend			0.99	0.72

[1]Adjusted for urinary creatinine
[2]Adjusted for age, education, smoking status, body mass index, total energy intake, sodium intake, and urinary creatinine

Table 4: HRs (95% CIs) for cardiovascular mortality by tertiles of urinary concentrations of total and individual phytoestrogens in the continuous National Health and Nutrition Examination Survey, 1999-2004

	Cardiovascular Mortality			
Phytoestrogens (ng/mL)	**No. of Cases**	**Person-Years**	**Creatinine-Adjusted HR (95% CI)[1]**	**Multivariable-Adjusted HR (95 % CI)[2]**
Total Phytoestrogens				
T1 (4-14)	42	3,035	Reference	Reference
T2 (415-1,047)	37	2,819	0.83 (0.47, 1.49)	0.58 (0.31, 1.09)
T3 (1,048-112,457)	29	2,231	0.80 (0.42, 1.53)	0.63 (0.31, 1.28)
p-trend			0.55	0.36
Isoflavones				
T1 (1-58)	31	2,346	Reference	Reference
T2 (59-219)	37	2,698	2.07 (1.09, 3.92)	1.97 (0.98, 3.97)
T3 (220-55,729)	40	3,041	1.96 (1.01, 3.82)	2.14 (1.03, 4.47)
p-trend			0.21	0.15
Genistein				
T1 (0-13)	33	2,455	Reference	Reference
T2 (14-54)	38	2,828	1.76 (0.95, 3.24)	1.59 (0.83, 3.06)
T3 (55-25,700)	37	2,802	1.70 (0.89, 3.22)	1.39 (0.69, 2.80)
p-trend			0.28	0.68
Daidzein				
T1 (0-25)	30	2,265	Reference	Reference
T2 (26-115)	38	2,819	1.66 (0.88, 3.15)	1.48 (0.74, 2.97)
T3 (116-29,200)	40	3,001	1.96 (1.02, 3.74)	2.05 (1.02, 4.11)
p-trend			0.10	0.06
Equol				
T1 (0-3)	36	2,629	Reference	Reference
T2 (4-11)	35	2,598	1.24 (0.67, 2.27)	1.40 (0.72, 2.74)
T3 (12-17,200)	29	2,220	0.95 (0.48, 1.86)	1.22 (0.57, 2.60)
p-trend			0.71	0.78
O-desmethylangolensin				
T1 (0-1)	32	2,374	Reference	Reference
T2 (2-9)	28	2,066	1.15 (0.60, 2.19)	1.07 (0.53, 2.15)
T3 (10-9,890)	40	3,025	1.50 (0.81, 2.77)	1.71 (0.87, 3.35)
p-trend			0.19	0.07
Lignans				
T1 (0-225)	40	2,858	Reference	Reference
T2 (226-691)	39	3,009	0.83 (0.47, 1.46)	0.55 (0.30, 1.02)
T3 (692-85,847)	29	2,218	0.73 (0.39, 1.38)	0.48 (0.24, 0.97)
p-trend			0.36	0.07
Enterodiol				
T1 (0-20)	38	2,805	Reference	Reference
T2 (21-63)	38	2,961	1.15 (0.65, 2.04)	1.36 (0.74, 2.48)
T3 (64-18,000)	30	2,177	0.92 (0.48, 1.77)	0.71 (0.87, 1.78)
p-trend			0.69	0.52
Enterolactone				
T1 (0-173)	40	2,880	Reference	Reference
T2 (174-595)	37	2,837	0.98 (0.56, 1.72)	0.68 (0.37, 1.26)
T3 (596-85,300)	31	2,368	0.78 (0.41, 1.48)	0.54 (0.27, 1.07)
p-trend			0.43	0.10

[1]Adjusted for urinary creatinine

[2]Adjusted for age, education, smoking status, body mass index, total energy intake, sodium intake, and urinary creatinine

Table 5: HRs (95% CIs) for all-cause mortality by tertiles of urinary concentrations of total and individual phytoestrogens in the continuous National Health and Nutrition Examination Survey, 1999-2004

	All-Cause Mortality			
Phytoestrogens (ng/mL)	**No. of Cases**	**Person-Years**	**Creatinine-Adjusted HR (95% CI)[1]**	**Multivariable-Adjusted HR (95 % CI)[2]**
Total Phytoestrogens				
T1 (4-14)	102	7,250	Reference	Reference
T2 (415-1,047)	100	7,269	1.06 (0.76, 1.47)	0.78 (0.55, 1.12)
T3 (1,048-112,457)	88	6,263	0.87 (0.60, 1.25)	0.69 (0.46, 1.02)
p-trend			0.36	0.09
Isoflavones				
T1 (1-58)	87	6,332	Reference	Reference
T2 (59-219)	100	7,119	1.46 (1.03, 2.08)*	1.34 (0.93, 0.95)
T3 (220-55,729)	103	7,331	1.26 (0.87, 1.83)	1.22 (0.82, 1.82)
p-trend			0.69	0.71
Genistein				
T1 (0-13)	87	6,325	Reference	Reference
T2 (14-54)	97	6,968	1.60 (1.13, 2.28)*	1.44 (1.00, 2.08)
T3 (55-25,700)	106	7,489	1.44 (1.00, 2.08)	1.17 (0.79, 1.74)
p-trend			0.31	0.97
Daidzein				
T1 (0-25)	82	5,948	Reference	Reference
T2 (26-115)	101	7,384	1.23 (0.86, 1.77)	1.09 (0.74, 1.60)
T3 (116-29,200)	107	7,450	1.44 (1.01, 2.07)*	1.43 (0.98, 2.08)
p-trend			0.07	0.47
Equol				
T1 (0-3)	93	6,666	Reference	Reference
T2 (4-11)	86	6,222	1.01 (0.70, 1.46)	1.06 (0.72, 1.56)
T3 (12-17,200)	86	6,067	1.07 (0.74, 1.55)	1.18 (0.79, 1.76)
p-trend			0.71	0.42
O-desmethylangolensin				
T1 (0-1)	91	6,630	Reference	Reference
T2 (2-9)	91	6,375	1.24 (0.87, 1.77)	1.12 (0.77 - 1.62)
T3 (10-9,890)	93	6,725	1.13 (0.79, 1.63)	1.12 (0.76, 1.65)
p-trend			0.90	0.72
Lignans				
T1 (0-225)	101	7,106	Reference	Reference
T2 (226-691)	112	8,281	1.26 (0.91, 1.74)	0.99 (0.70, 1.40)
T3 (692-85,847)	77	5,395	0.86 (0.60, 1.25)	0.65 (0.43, 0.96)
p-trend			0.26	0.019
Enterodiol				
T1 (0-20)	105	7,566	Reference	Reference
T2 (21-63)	93	6,877	0.92 (0.65, 1.29)	1.05 (0.73, 1.50)
T3 (64-18,000)	88	6,060	0.97 (0.68, 1.37)	0.98 (0.67, 1.43)
p-trend			0.95	0.85
Enterolactone				
T1 (0-173)	102	7,177	Reference	Reference
T2 (174-595)	110	8,141	1.34 (0.97, 1.85)	1.09 (0.77, 1.54)
T3 (596-85,300)	78	5,464	0.86 (0.59, 1.25)	0.65 (0.44, 0.97)
p-trend			0.22	0.014

[1]Adjusted for urinary creatinine

[2]Adjusted for age, education, smoking status, body mass index, total energy intake, sodium intake, and urinary creatinine

Chapter 3

Dietary Intake of Phytoestrogens and the Risk of Prostate Cancer in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO)

Abstract

Background: Prostate cancer is the most common non-cutaneous cancer and second leading cause of cancer death among men in the US. However, no effective preventive measures are available as few modifiable risk factors have been identified.

Objective: The present study aimed to investigate the associations between dietary intake of total and individual phytoestrogens and the risk of total and advanced prostate cancer among 30,097 participants in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO).

Design: During a median follow up of 11.5 years, 3,628 cases of prostate cancer (including 396 advanced cases) have been identified from 30,097 men. Dietary intake of phytoestrogens was assessed with a validated food frequency questionnaire. Cox proportional hazards regression was performed to estimate hazard ratios (HRs) and 95% confidence intervals (CI) for dietary phytoestrogens in relation to prostate cancer risk.

Results: An increased risk of advanced prostate cancer was found for the dietary intake of isoflavones (HR for quintile 5 vs. quintile 1: 1.58; 95% CI: 1.11, 2.24), genistein (HR for quintile 4 vs. quintile 1: 1.42; 95% CI: 1.02, 1.98), daidzein (HR for quintile 5 vs. quintile 1: 1.62; 95% CI: 1.13, 2.32) and glycitein (HR for quintile 5 vs. quintile 1: 1.53; 95% CI: 1.09, 2.15). Conversely, dietary intake of genistein was associated with a reduced risk of non-advanced prostate cancer (HR for quintile 5 vs. quintile 1: 0.88; 95% CI: 0.78, 0.99) and total prostate cancer (HR for quintile 3 vs. quintile 1: 0.90; 95% CI: 0.81, 1.00).

Conclusion: This national prospective cohort study revealed that dietary isoflavone intake modulated the risk of prostate cancer, and that this effect may differ by the aggressiveness of the disease.

Introduction

Primary prevention of chronic diseases such as cancer is one of the major goals of public health. Prostate cancer is the most common non-cutaneous cancer and the second leading cause of cancer death among men in the US, with 238,590 new cases and 29,720 deaths estimated to occur in 2013 (183). Most risk factors identified to date are non-modifiable, such as age, ethnicity, and family history (184). A modifiable factor that is potentially protective for prostate cancer but has not yet been well explored in the US population is dietary intake of total and individual phytoestrogens.

Phytoestrogens are a group of non-steroidal plant metabolites that abound in soy products, legumes, and chick peas (185, 186). Individual phytoestrogens that exist in these food products include genistein, daidzein, and glycitein (187), and to a lesser degree, formononetin, biochanin A, and coumestrol (54). Experimental studies suggest that phytoestrogen intake may modulate the risk of certain types of cancer (134, 135) due to their structural similarity to 17β-estradiol (188) and the resulting competitive binding to estrogen receptors (136, 189). Of particular relevance to prostate carcinogenesis, animal studies have shown that phytoestrogens reduce serum testosterone levels in rats (190, 191). As a significant positive association between serum testosterone and prostate cancer risk has been consistently reported in epidemiologic studies (192, 193), the results of these animal studies suggest that it is biologically plausible that high intake of phytoestrogens may reduce the risk of this malignancy.

Despite experimental evidence, it is still not clear whether there is an association between phytoestrogen intake and prostate cancer risk in Western populations. Results of

a few previous studies on this topic have been discrepant (103, 194). Despite a low average consumption of soy products in Western countries (195, 196), it is still feasible to investigate the effect of phytoestrogens on this population because of additional dietary sources of phytoestrogens (173-175) leading to a considerable between person variation in phytoestrogen intake (121, 197). To date, no epidemiologic studies have evaluated the associations between phytoestrogen intake and the development of total and advanced cases of prostate cancer in a large sample of the US population. Therefore, the present study investigated this research question using data on total and individual phytoestrogen intake, as well as total and advanced cases of prostate cancer, previously collected from the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO).

Subjects and Methods

Study Population

Data analyzed in this study were obtained from the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO). Approximately 155,000 participants were enrolled from 10 medical centers across the US between November 1993 and July 2001 in the PLCO. Study participants were followed up through December 31, 2009. Only male participants were eligible for the present study as the outcome of interest was prostate cancer. Further exclusion criteria included participants who did not complete a baseline questionnaire, those with a history of cancer, and those without follow-up after the enrollment period. After those exclusions, a total of 30,097 subjects met the eligibility criteria for this study. During a median follow up of 11.5 years, 3,628 cases of prostate cancer (including 396 advanced cases) were identified from the 30,097 men.

Advanced cases of prostate cancer were defined as stage II prostate cancer with a Gleason score of ≥ 8, and all stage III or stage IV cases (198). The date of randomization is considered to be the entry date into the trial (198). The PLCO is a de-identified, publicly released data set and thus it was determined to be exempt from human subjects review by the Indiana University IRB.

Data Collection

All eligible subjects were asked to complete a sex-specific baseline questionnaire and a food frequency questionnaire. The vast majority of (96.8%) of study participants completed the baseline questionnaire that solicited information on age, race, BMI, marital status, education level, daily physical activity, cigarette smoking, family history of prostate cancer, family history of other cancer, aspirin use, ibuprofen use, and vasectomy status (198).

The Dietary Questionnaire (DQX) was offered to the intervention arm participants at the time of randomization into the trial. This 137-item food frequency questionnaire was developed to assess usual diet and alcohol consumption during the previous year. The DQX also contained questions on use of vitamins and other dietary supplements. Dietary intake of energy and nutrients were calculated by multiplying the amount of energy and nutrients in a standard portion size of each food item by the reported frequency of consumption and summing over all food items. Examples of dietary and nutrient variables included total iron intake, caffeine, total dietary energy, processed meat consumption, and red meat consumption. Individual phytoestrogens considered for this study included genistein, daidzein, glycitein, coumestrol, formononetin, and biochanin A.

Total phytoestrogens were calculated by summing the individual phytoestrogens, with a similar calculation performed for total isoflavones. The nutrient amounts were based on values from national dietary databases including the USDA's Continuing Survey of Food Intakes by Individuals and the University of Minnesota's Nutrition Data Systems for Research (198).

Statistical Analysis

The study population was divided into quintiles for total and each individual phytoestrogen. Demographic, anthropometric, and lifestyle characteristics of subjects (including age, race/ethnicity, BMI, education, smoking status, alcohol intake, caffeine intake, red meat intake, and family history of prostate cancer) were compared across the total phytoestrogen intake (mg/day) quintiles (Q1: 0.0 – 0.29; Q2: 0.30 – 0.50; Q3: 0.51 – 0.77; Q4: 0.78 – 1.22; Q5: 1.23 – 68.66). Chi-square tests and analysis of variance (ANOVA) were used to compare differences in categorical and continuous variables among quintiles, respectively. Because of non-normal distributions of phytoestrogen intake, Wilcoxon Rank-Sum tests were used to make pair-wise comparisons of total and individual phytoestrogen intake between subjects with advanced prostate cancer and non-cases, as well as total subjects with any prostate cancer and non-cases.

Cox proportional hazards regression was performed to calculate hazard ratios (HRs) and 95% confidence intervals (CIs) for prostate cancer risk in relation to dietary phytoestrogens. Each outcome of interest was compared against the remaining total population, with no additional cases removed from the comparison. Separate analyses were performed on advanced and non-advanced prostate cancer cases since many non-

advanced cases would likely not progress into advanced disease. Dietary intake of total phytoestrogens and each individual phytoestrogen were divided into quintiles with the lowest quintile as the reference group to estimate HRs and 95% CIs for the upper four quintiles. Covariates in the multivariable models included age, race, BMI, smoking status, alcohol intake, and family history of prostate cancer. Other variables that were examined as potential confounders included education, marital status, daily physical activity, family history of other cancer, aspirin use, ibuprofen use, vasectomy, dietary iron, caffeine intake, dietary energy, processed meat consumption, and red meat consumption. None of these variables were ultimately included in the final model because they were not statistically significantly related to prostate cancer or did not substantially alter risk estimates (<10%). Several factors were tested for potential interactions with dietary phytoestrogen in relation to the development of prostate cancer, including age, race, BMI, education, smoking status, alcohol intake, caffeine intake, red meat intake, and family history of prostate cancer. None of these variables showed a statistically significant interaction and were therefore not included in the final model. All models met the assumptions for proportional hazards models. Additional Cox proportional hazards regression analyses were performed after removing subjects from the dataset who developed prostate cancer within two-years of enrollment to eliminate any effects arising from preexisting, undetected cases of prostate cancer (199, 200). SPSS version 20 was used for statistical analysis. A p-value of <0.05 was considered statistically significant.

Results

Characteristics of study subjects are shown in **Table 6**. Statistically significant differences existed in race, education, smoking status, alcohol intake, and family history of prostate cancer across total phytoestrogen quintiles. Subjects in the highest quintile of total dietary phytoestrogens were more likely to be Asian, better educated, and never smokers, were less likely to be non-Hispanic White, and had a lower BMI.

Median levels and interquartile ranges of dietary intake of total and individual phytoestrogens (mg/day) were compared between total prostate cancer cases, advanced cases, and those who had not developed prostate cancer through the censor date (December 31, 2009) (**Table 7**). Advanced cases of prostate cancer had statistically significantly higher intake of total phytoestrogens, isoflavones, genistein, daidzein, and glycitein than non-cases. Insignificant differences were observed for dietary intake of total and individual phytoestrogens between total cases of prostate cancer and non-cases.

HRs (95% CIs) for each of three prostate cancer outcomes considered in relation to dietary intake of total and individual phytoestrogens are presented in **Tables 8, 9, and 10**. After adjustment for confounders, a significantly increased risk of advanced prostate cancer was found for dietary intake of total isoflavones (HR for Q5 vs. Q1: 1.58; 95% CI: 1.11, 2.24), genistein (HR for Q4 vs. Q1: 1.42; 95% CI: 1.02, 1.98), daidzein (HR for Q5 vs. Q1: 1.62; 95% CI: 1.13, 2.32), and glycitein (HR for Q5 vs. Q1: 1.53; 95% CI: 1.09, 2.15). Conversely, dietary intake of genistein was significantly associated with a reduced risk of non-advanced prostate cancer (HR for Q5 vs. Q1: 0.88; 95% CI: 0.78, 0.99).

After removing subjects who developed prostate cancer within two-years of enrollment from analysis, an increased risk of advanced prostate cancer appeared for

subjects who were in the fifth quintile of total dietary phytoestrogens, and persisted for those in the fifth quintile of total dietary isoflavones, the fourth quintile of dietary genistein, the second, third, and fifth quintile of dietary daidzein, and the fifth quintile of dietary glycitein, but disappeared for those who were in the second quintile of formononetin. In addition, a reduced risk of non-advanced prostate cancer was no longer significant for subjects who were in the third and fifth quintile of dietary genistein and in the third quintile of dietary genistein in relation to total prostate cancer.

Discussion

The present study showed that higher dietary intake of isoflavones, genistein, daidzein, glycitein, and formononetin were significantly associated with an increased risk of advanced prostate cancer after adjustment for possible confounding variables. Conversely, higher dietary intake of genistein was significantly associated with a decreased risk of non-advanced prostate cancer and total prostate cancer in a large sample of the US population. The approach of dividing prostate cancer cases into separate groups of advanced and non-advanced cases is supported by the growing body of evidence that suggests that these different types of prostate cancer may not be etiologically similar (124).

Genistein is one of the most widely investigated phytoestrogen metabolites. The present study showed a significant increased risk between dietary genistein and advanced prostate cancer, but a significant decreased risk between dietary genistein and both non-advanced and total prostate cancer. The results of decreased risk of non-advanced and total prostate cancer are consistent with the findings from other studies that reported an

inverse association between plasma concentrations of genistein and the risk of prostate cancer (201, 202). Some experimental studies have also revealed a protective effect of genistein on prostate cancer (161, 203), although total phytoestrogens may be more effective than genistein alone for inhibiting the proliferation of prostate cancer (204). Recently, one experimental study conducted by Nakamura et al. revealed that genistein may play a role in increasing growth factor signaling and promoting tumor progression in advanced prostate cancer (84). In that study, a prostatectomy sample was grafted into mice, and increased lymph node and additional organ metastases was observed in the genistein-treated group compared with the control group. Further analysis by Nakamura et al. showed that the genistein-treated mice had more proliferating and fewer apoptotic cancer cells than the controls, leading to enhanced tumorigenic activity (84). The increased risk between dietary genistein and advanced prostate cancer that was observed in the present study has not been reported in other observational studies. The differences might arise from two reasons: 1) many previous studies have focused on Asian populations where the intake of phytoestrogens is higher than in Western populations; 2) in the other studies, total prostate cancer was examined, conversely in our study, risks were examined for advanced and non-advanced disease separately.

It is remains unclear why genistein simultaneously increased the risk of advanced prostate cancer and decreased the risk of non-advanced prostate cancer. There is a biologic plausibility for the increased risk of advanced prostate cancer observed in this study. It has been hypothesized that estrogen may play a role in prostate carcinogenesis due to the possible mutagenic effects of estrogen metabolites (205), and phytoestrogens can induce estrogenic responses in the body due to their structural similarity to 17β-

estradiol (12). In addition, certain repeat polymorphisms in genes involved in estrogen synthesis and metabolism have demonstrated an increased risk of prostate cancer, with some genotypes showing a significant odds ratio of greater than two for advanced prostate cancer (Gleason score ≥ 7). Interestingly, the largest significantly increased risk of advanced prostate was observed in patients who were treated with finasteride, which inhibits the conversion of testosterone to dihydrotestosterone, causing an increase of estrogen levels (206). Therefore, it is reasonable to assume that an increased intake of phytoestrogens exhibiting increased estrogenic activity would also be associated with an increased risk of advanced prostate cancer.

Daidzein, like genistein, is one of the main isoflavones found in soy products and has also been among the most widely investigated. The current study did not show a significant association of dietary daidzein intake with both non-advanced and total prostate cancer, which was consistent with the results of several other studies in which daidzein intake was not associated with prostate cancer (202, 207, 208). Two double blind, placebo controlled, randomized controlled trials also showed no significant association between daidzein supplementation and the risk of prostate cancer (209, 210). The increased risk between dietary daidzein and advanced prostate cancer that was seen in the present study was not observed in other studies. Since daidzein along with genistein are the two phytoestrogen metabolites found in the highest concentrations in commonly eaten foods, it remains biologically plausible that daidzein is also associated with an increased risk of advanced prostate cancer because of the estrogenic effects.

The present study has several advantages. The large sample size permitted the performance of separate analyses for both advanced and non-advanced prostate cancer.

The results of these analyses showed clear differences in the effects of phytoestrogens on the risk of indolent and aggressive prostate cancer. The prospective nature of the PLCO study largely excluded the possibility of reverse causality between intake of total and individual phytoestrogens and the risk of prostate cancer. Most previous investigations of the effect of phytoestrogens on cancer risk were small case-control studies (207, 211-213), which did not allow for the establishment of the temporal relationship.

There were several limitations of the present study that should be considered when interpreting the obtained results. Intake of total and individual phytoestrogens was estimated using a food frequency questionnaire. In addition to recall bias occurring in questionnaire-based dietary assessment, total amounts of phytoestrogens may be inadequately represented in many food composition databases. Soy additives are found in some processed foods (173), and certain isoflavones are naturally present in lower concentrations in other foods such as vegetables (214), fruits, and nuts (215). The isoflavones from these food products are most likely to be underrepresented in food composition databases. Another limitation of using a dietary questionnaire as opposed to urinary or plasma concentrations is that this method is unable to capture phytoestrogen metabolites produced by intestinal bacteria such as equol and O-desmethylangolensin (216). To better evaluate the effects of phytoestrogens on prostate cancer risk, it is preferable to assess dietary intake of phytoestrogens and their urinary biomarkers in the same populations. Another strength of utilizing phytoestrogen biomarkers is that they differ in their levels of biologic activity (195). Nevertheless, data on phytoestrogen biomarkers are not available from the PLCO study.

In summary, the present study suggests that higher dietary intake of isoflavones, genistein, daidzein, and glycitein were associated with an increased risk of development of advanced prostate cancer. Conversely, higher dietary intake of genistein was associated with a reduced risk of non-advanced, and total prostate cancer. Since many cases of non-advanced prostate cancer will have little impact on the patients' quality of life (217), it is important to determine modifiable factors that may increase the risk of advanced prostate cancer. If results reported in the present study are confirmed in other epidemiologic studies, modifying intake of phytoestrogens may offer innovative practical avenues for the prevention of prostate cancer.

Table 6: Baseline characteristics of study subjects by quintiles of dietary intake of total phytoestrogens (mg/day) in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO)

	Total Phytoestrogens (mg/day)					
Characteristics	**Q1 (0.0 - 0.29)**	**Q2 (0.30 - 0.50)**	**Q3 (0.51 - 0.77)**	**Q4 (0.78 - 1.22)**	**Q5 (1.23 - 68.66)**	
	n = 6,018	**n = 6,023**	**n = 6,015**	**n = 6,025**	**n = 6,016**	**p-value**
Age [Mean (SD)]	61.7 (5.26)	62.8 (5.24)	63.5 (5.19)	63.0 (5.31)	62.7 (5.35)	<0.001
Race/Ethnicity (%)						
Non-Hispanic White	20.6	20.7	20.7	20.8	17.1	<0.001
Non-Hispanic Black	25.3	21.5	20.8	16.2	16.1	
Asian	0.7	1.4	2.9	5.1	89.9	
Other	17.4	17.4	15.3	16.6	33.3	
BMI [Mean (SD)]	27.9 (4.20)	27.8 (4.06)	27.7 (4.05)	27.5 (4.09)	27.1 (4.22)	<0.001
Education (%)						
Less than High School	23.9	22.0	22.8	16.7	14.6	<0.001
High School Graduate or Equivalent	25.6	23.2	20.7	16.5	14.2	
Post High School Education	20.7	20.9	20.3	19.7	18.4	
College Graduate or Higher	16.2	17.6	18.9	22.5	24.8	
Smoking Status (%)						
Never Smoker	17.7	19.0	20.0	21.2	22.2	<0.001
Former Smoker	26.8	23.1	18.6	17.0	14.4	
Current Smoker	20.3	20.1	20.3	19.8	19.6	
Alcohol Intake (%)						
Drinkers	18.8	19.9	21.0	20.5	19.7	<0.001
Non-Drinkers l	25.2	20.5	15.3	17.8	21.1	
Caffeine Intake (mg/day) [Mean (SD)]	561.6 (665.67)	555.0 (647.55)	550.9 (647.99)	550.2 (629.44)	549.6 (611.52)	0.883
Red Meat Intake (g/day) [Mean (SD)]	40.1 (34.51)	44.3 (36.61)	43.6 (38.85)	44.5 (39.16)	39.6 (40.92)	<0.001
Family History of Prostate Cancer						
Yes	18.3	20.5	21.0	21.6	18.6	0.032
No	20.0	20.0	19.9	19.9	20.2	

Table 7: Differences in dietary intake of total and individual phytoestrogens (mg/day) between subjects who did and did not develop prostate cancer in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO)[1]

Phytoestrogens (mg/day)	Total Cases of Prostate Cancer (n = 3,628)	Advanced Prostate Cancer (n = 396)	Non-Cases (n = 26,469)	p-value: Total Cases vs. Non-Cases	p-value: Advanced vs. Non-Cases
Total Phytoestrogens	0.63 (0.35, 1.01)	0.69 (0.40, 1.12)	0.63 (0.34, 1.05)	0.81	0.008
Isoflavones	0.44 (0.22, 0.75)	0.49 (0.26, 0.83)	0.43 (0.21, 0.76)	0.72	0.002
Genistein	0.18 (0.05, 0.34)	0.20 (0.07, 0.36)	0.17 (0.05, 0.35)	0.51	0.007
Daidzein	0.26 (0.15, 0.40)	0.28 (0.17, 0.44)	0.25 (0.14, 0.41)	0.46	0.002
Glycitein	0.01 (0.00, 0.02)	0.01 (0.00, 0.03)	0.01 (0.00, 0.03)	0.32	<0.001
Coumestrol	0.08 (0.04, 0.16)	0.09 (0.04, 0.16)	0.08 (0.04, 0.17)	0.46	0.65
Formononetin	0.01 (0.01, 0.02)	0.01 (0.00, 0.02)	0.01 (0.01, 0.02)	0.38	0.11
Biochanin A	0.05 (0.03, 0.08)	0.05 (0.04, 0.08)	0.05 (0.03, 0.08)	0.08	0.06

[1] Values are medians (interquartile ranges)

Table 8: HRs (95% CIs) for advanced prostate cancer according to quintiles of total and individual phytoestrogen intake (mg/day) in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial

	Advanced Prostate Cancer Cases			
Phytoestrogens (mg/day)	**No. of Cases**	**Person Years**	**Crude HR (95% CI)**	**Adjusted HR (95% CI)[1]**
Total Phytoestrogens				
Q1 (0.0 - 0.29)	57	3,644	Reference	Reference
Q2 (0.30 - 0.50)	78	4,972	1.35 (0.96 - 1.89)	1.27 (0.90 - 1.81)
Q3 (0.51 - 0.77)	87	5,702	1.47 (1.06 - 2.06)*	1.29 (0.91 - 1.82)
Q4 (0.78 - 1.22)	85	5,446	1.46 (1.04 - 2.04)*	1.28 (0.90 - 1.81)
Q5 (1.23 - 68.66)	89	5,783	1.52 (1.09 - 2.11)*	1.41 (0.99 - 2.01)
p-trend			0.06	0.13
Isoflavones				
Q1 (0.0 - 0.18)	54	3,452	Reference	Reference
Q2 (0.19 - 0.34)	76	4,798	1.40 (0.99 - 1.99)	1.29 (0.91 - 1.85)
Q3 (0.35 - 0.54)	89	5,807	1.58 (1.13 - 2.22)*	1.30 (0.92 - 1.84)
Q4 (0.55 - 0.85)	82	5,295	1.48 (1.05 - 2.09)*	1.25 (0.88 - 1.79)
Q5 (0.86 - 66.31)	95	6,195	1.72 (1.23 - 2.40)*	1.58 (1.11 - 2.24)*
p-trend			0.010	0.028
Genistein				
Q1 (0.0 - 0.04)	59	3,836	Reference	Reference
Q2 (0.05 - 0.11)	72	4,495	1.21 (0.86 - 1.71)	1.19 (0.84 - 1.70)
Q3 (0.12 - 0.22)	88	5,706	1.43 (1.03 - 1.99)*	1.19 (0.85 - 1.68)
Q4 (0.23 - 0.39)	95	6,157	1.57 (1.13 - 2.17)*	1.42 (1.02 - 1.98)*
Q5 (0.40 - 37.07)	82	5,353	1.35 (0.96 - 1.88)	1.25 (0.87 - 1.78)
p-trend			0.16	0.29
Daidzein				
Q1 (0.0 - 0.12)	51	3,233	Reference	Reference
Q2 (0.13 - 0.20)	86	5,533	1.67 (1.18 - 2.36)*	1.57 (1.10 - 2.23)*
Q3 (0.21 - 0.31)	90	5,852	1.71 (1.21 - 2.41)*	1.50 (1.05 - 2.14)*
Q4 (0.32 - 0.47)	74	4,769	1.43 (1.00 - 2.04)	1.27 (0.88 - 1.83)
Q5 (0.48 - 27.18)	95	6,160	1.82 (1.29 - 2.56)*	1.62 (1.13 - 2.32)*
p-trend			0.017	0.11
Glycitein				
Q1 (0.0 - 0.001)	63	4,028	Reference	Reference
Q2 (0.002 - 0.003)	69	4,349	1.04 (0.74 - 1.47)	1.11 (0.78 - 1.57)
Q3 (0.004 - 0.010)	87	5,646	1.50 (1.09 - 2.08)*	1.38 (0.99 - 1.93)
Q4 (0.011 - 0.030)	85	5,632	1.50 (1.08 - 2.07)*	1.28 (0.91 - 1.81)
Q5 (0.031 - 3.130)	91	5,892	1.51 (1.09 - 2.08)*	1.53 (1.09 - 2.15)*
p-trend			0.021	0.027
Coumestrol				
Q1 (0.0 - 0.03)	75	4,894	Reference	Reference
Q2 (0.04 - 0.07)	73	4,690	1.01 (0.73 - 1.39)	1.02 (0.73 - 1.42)
Q3 (0.08 - 0.11)	86	5,582	1.18 (0.87 - 1.61)	1.13 (0.82 - 1.56)
Q4 (0.12 - 0.20)	85	5,409	1.16 (0.85 - 1.58)	1.14 (0.83 - 1.57)
Q5 (0.21 - 2.19)	77	4,972	1.07 (0.78 - 1.46)	1.05 (0.76 - 1.45)
p-trend			0.80	0.89
Formononetin				
Q1 (0.0 - 0.006)	77	4,952	Reference	Reference
Q2 (0.007 - 0.010)	93	6,055	1.46 (1.08 - 1.97)*	1.42 (1.04 - 1.93)*
Q3 (0.011 - 0.014)	69	4,404	1.29 (0.93 - 1.78)	1.26 (0.90 - 1.75)
Q4 (0.015 - 0.020)	74	4,802	1.26 (0.92 - 1.74)	1.21 (0.87 - 1.67)
Q5 (0.021 - 0.240)	83	5,334	1.42 (1.04 - 1.93)*	1.30 (0.95 - 1.79)
p-trend			0.12	0.34
Biochanin A				
Q1 (0.0 - 0.030)	69	4,498	Reference	Reference
Q2 (0.031 - 0.040)	74	4,771	1.11 (0.80 - 1.55)	1.08 (0.78 - 1.51)
Q3 (0.041 - 0.060)	83	5,378	1.24 (0.90 - 1.71)	1.16 (0.84 - 1.61)
Q4 (0.061 - 0.090)	95	6,119	1.44 (1.05 - 1.96)*	1.31 (0.95 - 1.80)
Q5 (0.091 - 0.590)	75	4,781	1.14 (0.82 - 1.59)	1.03 (0.74 - 1.44)
p-trend			0.36	0.84

[1]Adjusted for age, race/ethnicity, BMI, smoking status, alcohol intake, and family history of prostate cancer

Table 9: HRs (95% CIs) for non-advanced prostate cancer according to quintiles of total and individual phytoestrogen intake (mg/day) in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial

	Non-Advanced Prostate Cancer Cases			
Phytoestrogens (mg/day)	**No. of Cases**	**Person Years**	**Crude HR (95% CI)**	**Adjusted HR (95% CI)[1]**
Total Phytoestrogens				
Q1 (0.0 - 0.29)	627	39,110	Reference	Reference
Q2 (0.30 - 0.50)	674	42,683	1.07 (0.96 - 1.19)	1.03 (0.92 - 1.15)
Q3 (0.51 - 0.77)	674	43,104	1.06 (0.95 - 1.18)	0.98 (0.88 - 1.10)
Q4 (0.78 - 1.22)	672	42,733	1.06 (0.95 - 1.18)	1.00 (0.89 - 1.12)
Q5 (1.23 - 68.66)	585	37,191	0.92 (0.82 - 1.03)	0.95 (0.85 - 1.07)
p-trend			0.022	0.30
Isoflavones				
Q1 (0.0 - 0.18)	633	39,466	Reference	Reference
Q2 (0.19 - 0.34)	658	41,264	1.04 (0.94 - 1.16)	1.03 (0.92 - 1.15)
Q3 (0.35 - 0.54)	654	42,377	1.02 (0.92 - 1.14)	0.92 (0.82 - 1.03)
Q4 (0.55 - 0.85)	697	44,220	1.09 (0.98 - 1.22)	1.03 (0.92 - 1.15)
Q5 (0.86 - 66.31)	590	37,494	0.92 (0.82 - 1.03)	0.95 (0.85 - 1.07)
p-trend			0.08	0.50
Genistein				
Q1 (0.0 - 0.04)	670	42,006	Reference	Reference
Q2 (0.05 - 0.11)	637	39,702	0.95 (0.85 - 1.05)	0.94 (0.84 - 1.04)
Q3 (0.12 - 0.22)	663	42,935	0.97 (0.87 - 1.08)	0.87 (0.78 - 0.97)*
Q4 (0.23 - 0.39)	689	43,897	1.02 (0.92 - 1.13)	0.95 (0.85 - 1.06)
Q5 (0.40 - 37.07)	573	36,281	0.84 (0.75 - 0.94)*	0.88 (0.78 - 0.99)*
p-trend			0.005	0.14
Daidzein				
Q1 (0.0 - 0.12)	620	38,682	Reference	Reference
Q2 (0.13 - 0.20)	670	42,386	1.08 (0.97 - 1.21)	1.04 (0.93 - 1.16)
Q3 (0.21 - 0.31)	662	42,388	1.06 (0.95 - 1.18)	0.98 (0.87 - 1.09)
Q4 (0.32 - 0.47)	663	42,014	1.07 (0.96 - 1.19)	1.01 (0.90 - 1.13)
Q5 (0.48 - 27.18)	617	39,351	0.99 (0.88 - 1.10)	1.01 (0.90 - 1.14)
p-trend			0.39	0.95
Glycitein				
Q1 (0.0 - 0.001)	667	41,809	Reference	Reference
Q2 (0.002 - 0.003)	702	43,885	1.01 (0.91 - 1.112)	1.04 (0.94 - 1.16)
Q3 (0.004 - 0.010)	635	40,698	1.06 (0.95 - 1.18)	1.00 (0.89 - 1.12)
Q4 (0.011 - 0.030)	665	43,068	1.13 (1.02 - 1.26)*	1.03 (0.92 - 1.15)
Q5 (0.031 - 3.130)	563	35,361	0.89 (0.80 - 1.00)*	0.96 (0.86 - 1.08)
p-trend			0.012	0.31
Coumestrol				
Q1 (0.0 - 0.03)	672	42,560	Reference	Reference
Q2 (0.04 - 0.07)	632	39,921	0.98 (0.88 - 1.09)	0.96 (0.86 - 1.07)
Q3 (0.08 - 0.11)	678	42,725	1.04 (0.94 0 1.16)	1.03 (0.92 - 1.14)
Q4 (0.12 - 0.20)	631	40,196	0.96 (0.86 - 1.07)	0.94 (0.84 - 1.05)
Q5 (0.21 - 2.19)	619	39,419	0.95 (0.85 - 1.06)	0.96 (0.86 - 1.07)
p-trend			0.26	0.41
Formononetin				
Q1 (0.0 - 0.006)	777	49,098	Reference	Reference
Q2 (0.007 - 0.010)	682	42,934	1.06 (0.96 - 1.18)	1.06 (0.95 - 1.17)
Q3 (0.011 - 0.014)	553	34,885	1.03 (0.92 - 1.15)	1.01 (0.90 - 1.13)
Q4 (0.015 - 0.020)	608	38,814	1.03 (0.93 - 1.15)	1.00 (0.89 - 1.11)
Q5 (0.021 - 0.240)	612	39,090	1.04 (0.93 - 1.15)	1.01 (0.91 - 1.13)
p-trend			0.69	0.90
Biochanin A				
Q1 (0.0 - 0.030)	668	42,067	Reference	Reference
Q2 (0.031 - 0.040)	610	38,502	0.95 (0.86 - 1.06)	0.92 (0.82 - 1.03)
Q3 (0.041 - 0.060)	655	41,467	1.02 (0.92 - 1.14)	0.99 (0.89 - 1.10)
Q4 (0.061 - 0.090)	638	40,557	1.00)0.90 - 1.12)	0.94 (0.85 - 1.06)
Q5 (0.091 - 0.590)	661	42,228	1.04 (0.94 - 1.16)	0.99 (0.89 - 1.11)
p-trend			0.24	0.78

[1]Adjusted for age, race/ethnicity, BMI, smoking status, alcohol intake, and family history of prostate cancer

Table 10: HRs (95% CIs) for all prostate cancer according to quintiles of total and individual phytoestrogen intake (mg/day) in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial

	Total Prostate Cancer Cases			
Phytoestrogens (mg/day)	**No. of Cases**	**Person Years**	**Crude HR (95% CI)**	**Adjusted HR (95% CI)[1]**
Total Phytoestrogens				
Q1 (0.0 - 0.29)	684	42,754	Reference	Reference
Q2 (0.30 - 0.50)	752	47,655	1.09 (0.99 - 1.21)	1.05 (0.94 - 1.16)
Q3 (0.51 - 0.77)	761	48,806	1.10 (0.99 - 1.22)	1.01 (0.90 - 1.12)
Q4 (0.78 - 1.22)	757	48,179	1.10 (0.99 - 1.21)	1.02 (0.92 - 1.14)
Q5 (1.23 - 68.66)	674	42,974	0.97 (0.87 - 1.08)	0.99 (0.89 - 1.11)
p-trend			0.12	0.63
Isoflavones				
Q1 (0.0 - 0.18)	687	42,918	Reference	Reference
Q2 (0.19 - 0.34)	734	46,062	1.07 (0.97 - 1.19)	1.05 (0.94 - 1.17)
Q3 (0.35 - 0.54)	743	48,184	1.07 (0.96 - 1.18)	0.95 (0.85 - 1.06)
Q4 (0.55 - 0.85)	779	49,515	1.12 (1.02 - 1.25)	1.05 (0.94 - 1.17)
Q5 (0.86 - 66.31)	685	43,689	0.98 (0.89 - 1.09)	1.01 (0.90 - 1.12)
p-trend			0.43	0.94
Genistein				
Q1 (0.0 - 0.04)	729	45,842	Reference	Reference
Q2 (0.05 - 0.11)	709	44,197	0.97 (0.87 - 1.07)	0.96 (0.86 - 1.06)
Q3 (0.12 - 0.22)	751	48,641	1.01 (0.91 - 1.12)	0.90 (0.81 - 1.00)*
Q4 (0.23 - 0.39)	784	50,054	1.06 (0.96 - 1.18)	0.99 (0.89 - 1.10)
Q5 (0.40 - 37.07)	655	41,634	0.88 (0.79 - 0.98)*	0.91 (0.81 - 1.02)
p-trend			0.029	0.29
Daidzein				
Q1 (0.0 - 0.12)	671	41,915	Reference	Reference
Q2 (0.13 - 0.20)	756	47,919	1.13 (1.02 - 1.25)*	1.08 (0.97 - 1.20)
Q3 (0.21 - 0.31)	752	48,240	1.11 (1.00 - 1.23)	1.02 (0.91 - 1.13)
Q4 (0.32 - 0.47)	737	46,783	1.10 (0.99 - 1.22)	1.03 (0.92 - 1.14)
Q5 (0.48 - 27.18)	712	45,511	1.05 (0.95 - 1.17)	1.06 (0.95 - 1.18)
p-trend			0.98	0.56
Glycitein				
Q1 (0.0 - 0.001)	730	45,837	Reference	Reference
Q2 (0.002 - 0.003)	771	48,234	1.01 (0.91 - 1.12)	1.05 (0.95 - 1.16)
Q3 (0.004 - 0.010)	722	46,344	1.10 (0.99 - 1.22)	1.03 (0.93 - 1.15)
Q4 (0.011 - 0.030)	750	48,700	1.16 (1.05 - 1.29)*	1.05 (0.95 - 1.17)
Q5 (0.031 - 3.130)	654	41,253	0.95 (0.85 - 1.05)	1.01 (0.91 - 1.13)
p-trend			0.11	0.81
Coumestrol				
Q1 (0.0 - 0.03)	747	47,454	Reference	Reference
Q2 (0.04 - 0.07)	705	44,611	0.98 (0.88 - 1.09)	0.96 (0.87 - 1.07)
Q3 (0.08 - 0.11)	764	48,307	1.06 (0.96 - 1.17)	1.04 (0.93 - 1.15)
Q4 (0.12 - 0.20)	716	45,605	0.98 (0.89 - 1.09)	0.96 (0.87 - 1.07)
Q5 (0.21 - 2.19)	696	44,391	0.96 (0.87 - 1.07)	0.97 (0.87 - 1.07)
p-trend			0.33	0.47
Formononetin				
Q1 (0.0 - 0.006)	854	54,050	Reference	Reference
Q2 (0.007 - 0.010)	775	48,989	1.10 (0.99 - 1.21)	1.09 (0.99 - 1.20)
Q3 (0.011 - 0.014)	622	39,289	1.05 (0.95 - 1.17)	1.03 (0.93 - 1.14)
Q4 (0.015 - 0.020)	682	43,616	1.05 (0.95 - 1.17)	1.01 (0.91 - 1.12)
Q5 (0.021 - 0.240)	695	44,424	1.07 (0.97 - 1.18)	1.04 (0.94 - 1.15)
p-trend			0.37	0.84
Biochanin A				
Q1 (0.0 - 0.030)	737	46,565	Reference	Reference
Q2 (0.031 - 0.040)	684	43,273	0.97 (0.87 - 1.08)	0.93 (0.84 - 1.04)
Q3 (0.041 - 0.060)	738	46,845	1.04 (0.94 - 1.16)	1.00 (0.90 - 1.11)
Q4 (0.061 - 0.090)	733	46,676	1.05 (0.94 - 1.16)	0.98 (0.88 - 1.09)
Q5 (0.091 - 0.590)	736	47,009	1.05 (0.95 - 1.17)	1.00 (0.90 - 1.10)
p-trend			0.16	0.74

[1]Adjusted for age, race/ethnicity, BMI, smoking status, alcohol intake, and family history of prostate cancer

Chapter 4

Association between Urinary Phytoestrogens and C-Reactive Protein in the Continuous National Health and Nutrition Examination Survey

Abstract

Serum C-reactive protein (CRP) is an inflammatory biomarker. The potential protective effect of phytoestrogen intake on cancer risk may be mediated in part through its influence on serum CRP levels. This study is among the first to examine the associations between urinary concentrations of total and individual phytoestrogens and serum concentrations of CRP among 6,009 subjects aged ≥40 years in the continuous National Health and Nutrition Examination Survey (1999-2010). Phytoestrogen concentrations in spot urine (ng/mL) were measured using high performance liquid chromatography (HPLC) with tandem mass spectrometric (MS/MS) detection. Serum CRP levels (mg/L) were quantified by latex-enhanced nephelometry. After adjustment for urinary creatinine and other confounders, both linear and logistic regression analyses showed a significant inverse association between urinary excretion of total and all individual phytoestrogens and serum levels of CRP. Total phytoestrogens (β: -0.11; 95% CI: -0.13, -0.09) were associated with the largest reduction in CRP levels, followed by total lignans (β: -0.08; 95% CI: -0.10, -0.06) and enterolactone (β: -0.07; 95% CI: -0.08, -0.05). A reduced risk of developing high concentrations of CRP (≥ 3.0 mg/L) was most pronounced for enterolactone (OR for quartile 4 vs. quartile 1: 0.59; 95% CI: 0.51, 0.69), followed by total phytoestrogens (OR for quartile 4 vs. quartile 1: 0.63; 95% CI: 0.53, 0.73) and lignans (OR for quartile 4 vs. quartile 1: 0.64; 95% CI: 0.54, 0.75). In summary, dietary intake of total and individual phytoestrogens, assessed by measuring their urinary biomarkers, reduced the concentrations of CRP in a large, nationally representative sample of the US population.

Introduction

C-reactive protein (CRP) is a ring-shaped protein found in the plasma. It is the classical acute phase reactant, with the concentration rising rapidly in a cytokine-mediated response due to tissue injury, infection, or inflammation. Serum CRP levels are routinely measured to detect and monitor many human diseases (218). In past studies, elevated CRP levels have been associated with both cancer (219, 220) and cardiovascular disease (221, 222). A study using the Women's Health Initiative population showed that CRP levels are elevated before vascular events (223), and other studies have indicated that the use of oral estrogen may increase CRP levels as well (224). Inflammation has been linked to carcinogenesis (225) and certain inflammatory diseases have been associated with an increased risk of cancer through such mechanisms as the inhibition of apoptosis (226), prolonged activation of signal transducer and activator of transcription 3 (STAT3) (227), and the deactivation of tumor necrosis factor alpha (TNF-α) (228). In-vitro and animal studies have indicated that CRP may be an active participant in plaque development through such mechanisms as monocyte adhesion to the endothelium (229) and macrophage cholesterol accumulation (230).

Where CRP has been linked to an increase in the risk of chronic conditions, phytoestrogens, a group of compounds found in plants with a structural similarity to estrogen (12), have been associated with a decreased risk (168, 169). The biological effects seen in epidemiologic and experimental studies due to the consumption of phytoestrogens is caused by their competitive binding to estrogen receptors (136, 137). There are two principal classes of phytoestrogens, isoflavones and lignans. The most

important dietary source of isoflavones is soy products (43, 125), whereas dietary lignans primarily originate in flax seed (69). Both isoflavones and lignans are metabolized in the gut bacteria to form additional compounds that can act on the human body through competitive binding (76, 128).

Intake of total and individual phytoestrogens and their biomarkers have been associated with a decreased risk of both cancer and cardiovascular disease (132, 133), however the underlying mechanisms for these associations remain unclear. Studies conducted on the effects of phytoestrogens and CRP have shown mixed results, with some studies showing certain individual phytoestrogens may reduce CRP levels (115), and others showing no effect on CRP (231). However, many of these studies were conducted on specific groups of subjects such as postmenopausal women. Little is known about the association between phytoestrogens and CRP in a larger, more diverse sample of subjects. To date, no epidemiologic studies have evaluated the association between phytoestrogen intake and CRP levels in a nationally representative sample of the US population. Therefore, the present study investigated this research question using data on urinary excretion of total and individual phytoestrogens and serum CRP concentrations, previously collected from the continuous National Health and Nutrition Examination Survey (NHANES).

Subjects and Methods

Study Population

Data analyzed in this study were obtained from the NHANES for the years 1999-2010. This data source was selected because the collection of urinary phytoestrogen data

began in 1999. NHANES is an annual cross-sectional study initiated in the current form in 1999 by the Center for Disease Prevention and Control (CDC) to assess the health and nutritional status of the general US population. Data collection and sampling procedures for NHANES have been described in detail elsewhere (151).

From 1999 to 2010, a total of 62,160 individuals who enrolled in the NHANES also completed the interview and health examination components. As the objective of the present study is to investigate the association between urinary phytoestrogens and CRP and the relationship to cancer and cardiovascular disease, the analyses were confined to subjects who were 40 years of age or older. Urinary concentrations of phytoestrogens and CRP levels were measured only among a subsample of total NHANES participants. Subsampling in NHANES was performed to reduce participant burden and facilitate scheduling and completion of examinations. All subjects in the subsamples were randomly selected from the pool of total participants to obtain a nationally representative sample, with subsample weights calculated to account for the probability of being selected into the subsample and additional non-response (152). Excluding subjects who were <40 years of age, or without data on urinary phytoestrogens and CRP, left 6,009 subjects in the study for analysis. The de-identified data analyzed in the present study are freely available in public domains, and the approval for such data analysis by the Institutional Review Board of Indiana University was sought but determined not to be applicable.

Baseline Data Collection

NHANES participants were interviewed to collect data on demographic and lifestyle variables. Demographic variables used in this study included age, sex, race (non-Hispanic white, non-Hispanic black, and other race including multiracial), and education level (less than high school, high school graduate or equivalent, and more than high school). Lifestyle variables relevant to this study include smoking status [never smokers (smoking 0 to <100 cigarettes in lifetime), former smokers (smoking ≥100 cigarettes in lifetime but not currently smoking), and current smoker], alcohol consumption (0 drink/week, <1 drink/week, and >1 drink/week), and nutrient intake through a 24-hour food recall. Body mass index (BMI defined as kg/m^2) was calculated from height and weight measured during the medical examination portion of data collection.

Urinary Phytoestrogen Measurement

Phytoestrogen biomonitoring was accomplished by measuring urinary excretion of isoflavones (including daidzein, genistein, equol, and O-desmethylangolensin) and lignans (including enterodiol, and enterolactone) using high performance liquid chromatography (HPLC) with tandem mass spectrometric (MS/MS) detection (153). The methods for the collection and analysis of urine samples for phytoestrogen concentrations have been described in detail elsewhere (154). Briefly, spot urine specimens were collected at the Mobile Examination Centers the morning after a recommended fast, processed, stored at -20^0C, and then shipped to the Division of Environmental Health Laboratory Sciences at the NCHS for analysis. Urine samples were amended with stable isotope-labeled internal standards to improve method accuracy and precision, incubated

with a deconjugation enzyme to allow the quantification of individual phytoestrogens, extracted using solid phase extraction to remove interferences and improve sensitivity, and then analyzed using negative ion mode electrospray ionization HPLC-MS/MS, an assay with a high degree of specificity for each analyte (154). Previous studies have shown that laboratory measurement of phytoestrogen concentrations in the urine are a reliable biomarker of phytoestrogen intake (131, 148).

C-reactive protein

The level of CRP was determined by analyzing a blood specimen collected from the subjects. The methods for the collection and analysis of blood samples for CRP have been described in detail elsewhere (232). Briefly, blood samples were collected, processed, stored frozen at -20^{o} C or less, and shipped to the University of Washington in Seattle for analysis. CRP was quantified using latex-enhanced nephelometry. Particle enhanced assays were based on the reaction between a soluble analyte and the corresponding antigen or antibody bound to polystyrene particles. Particles consisting of a polystyrene core and a hydrophilic shell were used to link anti-CRP antibodies covalently. A diluted solution of each test sample was mixed with latex particles coated with mouse monoclonal anti-CRP antibodies. CRP present in the test sample forms an antigen-antibody complex with the latex particles. CRP concentrations were calculated by using a calibration curve. Data reduction of the signals was performed using a storable logit-log function for the calibration curve. These assays were performed on a Behring Nephelometer for quantitative CRP determination (232).

Statistical analysis

Sample weights were applied to the data through the calculation of a twelve-year weight variable according to the guidelines from the National Center for Health Statistics (NCHS) when combining two or more two-year cycles of the continuous NHANES data, to produce an unbiased national estimate. Total phytoestrogens were calculated by summing all of the individual phytoestrogens, with a similar calculation completed for both total isoflavones and total lignans. Demographic, anthropometric, lifestyle characteristics of subjects (including age, gender, race, BMI, education, smoking status, and alcohol intake) and total and individual phytoestrogen urinary concentrations (ng/mL) were compared by the quartiles of CRP level (mg/L) (Q1: 0.1 – 0.8; Q2: 0.9 – 2.1; Q3: 2.2 – 4.9; Q4: 5.0 – 296.0). Chi-square tests and analysis of variance were employed to compare differences in categorical and continuous variables among quartiles, respectively.

Multiple linear regression analyses were performed to determine the association between total and individual phytoestrogens and CRP levels. Urinary phytoestrogens and CRP levels were log-transformed for statistical analyses. Additionally, binary logistic regression analyses were used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for having a CRP of 3.0 mg/L or greater associated with quartiles of urinary phytoestrogens. Previous studies have shown that CRP levels > 3.0 mg/L are associated with an increased risk of cancer (233) and cardiovascular disease (234). For the logistic regression analyses, each urinary phytoestrogen was divided into quartiles (with the first quartile used as the reference group) to determine the association with higher CRP levels ($\geq$ 3.0 mg/L). The variables adjusted in the multivariable models of

both the linear and logistic regressions were age, gender, race, education, BMI, smoking status, alcohol intake, and urinary creatinine. Urinary creatinine was included to adjust for the variation in urinary dilution, which is a commonly used method (148, 177). Three separate models were analyzed, Model 1: adjusted for creatinine level; Model 2: further adjusted for age, gender, and race; and Model 3: further adjusted for education, BMI, smoking status, and alcohol intake. No interactions were found to be statistically significant, and thus no interaction terms were included in the final models. Factors that were tested for their interactions with urinary phytoestrogens in relation to CRP concentrations included age, gender, BMI, education, smoking status, total energy intake, and sodium intake. Marital status, and intake of alcohol, total energy, sodium, fat, and calcium were examined as potential confounders but not included in the final models because they were not statistically significant or did not substantively alter risk estimates (<10%). Two-sided p-values of <0.05 were considered statistically significant. SPSS version 21 was used for all statistical analyses.

Results

Characteristics of study subjects by CRP quartile are shown in **Table 11**. Subjects were statistically significantly different across CRP quartiles for age, gender, race, education, smoking status, and alcohol intake. Those in the highest quartile of CRP were more likely to be female, non-Hispanic black, obese, current smokers and non-drinkers, but were less likely to be in the higher education group. For the comparison of total and individual phytoestrogens, those in the highest quartile of CRP had statistically significantly decreased urinary concentrations of all phytoestrogens measurements, with

the exception of equol and enterodiol, which did not show significant differences between the quartiles.

Table 12 shows the results of the multiple linear regression. These analyses show that an increased level of each of the total and individual phytoestrogens were associated with a decrease in CRP levels, as all of the β coefficients in each model are negative and statistically significant. In the full model (Model 3), total phytoestrogens were associated with the largest decrease in CRP levels (β: -0.11; 95% CI: -0.13, -0.09), followed by total lignans (β: -0.08; 95% CI: -0.10, -0.06) and enterolactone (β: -0.07; 95% CI: -0.08, -0.05), respectively.

Table 13 shows the results of the binary multiple logistic regression analyses. The numbers of subjects in each phytoestrogen quartile with CRP levels ≥ 3.0 mg/L and < 3.0 mg/L are displayed in the table. The highest number of subjects with CRP levels ≥ 3.0 mg/L was associated with the first quartile of each phytoestrogen measurement, except for genistein and daidzein, which showed the highest number of subjects in the second quartile. Similar to the results from the linear regression, higher urinary concentrations of each of the total and individual phytoestrogens appeared to have lower concentrations of CRP in all three models. In the full model, the largest decreased odds of having a CRP level ≥ 3.0 mg/L was observed for enterolactone (OR for quartile 4 vs. quartile 1: 0.59; 95% CI: 0.51, 0.69), followed by total phytoestrogens (OR for quartile 4 vs. quartile 1: 0.63; 95% CI: 0.53, 0.73) and lignans (OR for quartile 4 vs. quartile 1: 0.64; 95% CI: 0.54, 0.75). The decreased odds for having a CRP level ≥ 3.0 mg/L for all urinary phytoestrogen measurements ranged from 13% to 41% in the highest quartile of the full model.

Discussion

The present study investigated the associations between urinary phytoestrogens and CRP levels using data collected from a nationally representative sample of the US population. It was found in both the linear and logistic regression analyses that urinary concentrations of total and individual phytoestrogens were significantly and inversely associated with CRP levels.

The results of this study are consistent with the results of several other studies in which an increased intake of particular phytoestrogens were also associated with a decrease in CRP (115, 235-237). A randomized, double-blind, placebo-controlled, dietary intervention crossover trial conducted by Hall et al. found that isoflavones have beneficial effects on CRP concentrations, but did not have the same effects on other inflammatory biomarkers that are associated with cardiovascular disease. The group receiving the isoflavones showed reduced CRP concentrations compared to those receiving the placebo (OR: 0.43; 95% CI: 0.27, 0.69) (115). Another randomized, double-blinded, crossover study also showed a decrease in CRP levels with higher levels of phytoestrogens as well (236).

Two epidemiological studies reported that an increase in phytoestrogen intake through dietary supplementation did not alter CRP levels (231, 238). These two studies that observed no association between phytoestrogen intake and CRP levels were conducted on very specific groups of people, with one study conducted on only postmenopausal women (231) and the other on a small group that included postmenopausal women and hypercholesterolemic men (238), which may explain the lack of association that was observed. The results of the current study should be more

generalizable since it included all adults over forty years of age in a nationally representative sample of the US.

There are several possible explanations to the present study's observed reduction of CRP levels associated with high phytoestrogen levels. Previous studies have shown that CRP levels may be reduced in the presence of increased levels of antioxidants (239, 240). Phytoestrogens have been shown to have antioxidant properties (241, 242), which may act to reduce the levels of circulating CRP. Additionally, studies have shown that higher levels of circulating estrogen due to orally delivered estrogen therapy are associated with increased levels of plasma CRP (224, 243, 244). Phytoestrogens are weak estrogens, with a much higher concentration needed to produce an equivalent biologic response (36). It is possible that competitive binding of phytoestrogens to estrogen receptors may decrease the effects of natural or pharmacological estrogens on CRP and therefore alter the risk of associated diseases.

The present study has several advantages. This was a large study conducted on a nationally representative sample of men and women in the US population. Large epidemiologic studies on the association between phytoestrogens and CRP levels in this type of national sample are non-existent. Most studies to this point have been small trials on specific groups of people (post-menopausal women), which reduces the generalizability of their results. Additionally, total and individual phytoestrogens as well as CRP levels were measured through laboratory methods, which is free of the recall errors that are inherent when food frequency data are used to assess phytoestrogen intake. Urinary excretion of phytoestrogens also provides a more accurate representation of phytoestrogen intake from all sources, compared to a food frequency questionnaire.

Another advantage of measuring urinary phytoestrogens is the measurement of each individual phytoestrogen as well as their metabolites that are produced by the intestinal bacteria but not associated with dietary intake, such as equol and O-desmethylangolensin (71). Accurate representations of individual phytoestrogens are important to determine their association with biomarkers such as CRP, as represented in the findings of this study that showed a decreased odds of high CRP levels for individual phytoestrogens ranging from 13% to 41%. This is also true for the association with chronic conditions as specific phytoestrogens differ in their levels of biological activity (135).

Limitations of the present study need to be considered in the interpretation of the obtained results. This study was a cross-sectional design which only allows for the capture of information from one point in time. This may not accurately reflect an individuals' usual urinary output of phytoestrogens due to within-person variation. Longer-term urinary output measures of phytoestrogens were not available from NHANES due to feasibility limitations of the study. The use of spot urine to determine phytoestrogen concentrations could be seen as a potential limitation of this study due to variation in urinary dilution. To adjust for this variation, each phytoestrogen concentration was normalized to urinary creatinine, a commonly used method (148, 177), since creatinine is excreted by glomerular filtration at a relatively constant rate (178). Additionally, phytoestrogen concentrations in spot urine, particularly for individual isoflavones, have been reported to be statistically significantly correlated with their concentrations measured in serum (179). Finally, a rise in CRP levels has been associated with infections (245) as well as traumatic injuries (246). Data on these

variables were not available from NHANES and therefore not controlled for in the multivariable analyses.

In summary, the present study suggests that higher urinary concentrations of total and individual phytoestrogens were associated with reduced concentrations of CRP, with the largest reduction observed in total phytoestrogens, lignans, and enterolactone. Increased CRP levels have been associated with an increased risk of certain chronic conditions, such as cancer (219, 220) and cardiovascular disease (229, 230), whereas increased phytoestrogen intake has been associated with a decreased risk of these conditions (168, 169). It is important and timely to further investigate the associations of CRP levels with phytoestrogen intake, its biomarkers, and metabolic polymorphisms as it is possible that the association between phytoestrogens and cancer or cardiovascular disease may be mediated through the influence of phytoestrogen intake on circulating levels of CRP.

Table 11: Baseline characteristics of subjects by quartiles of C-reactive protein (mg/L) in the continuous National Health and Nutrition Examination Survey, 1999-2010

	C-reactive protein (mg/L)				
Characteristics	**Quartile 1** **0.1 - 0.8** **n = 1,801**	**Quartile 2** **0.9 - 2.1** **n = 1,847**	**Quartile 3** **2.2 - 4.9** **n = 1,777**	**Quartile 4** **5.0 - 296.0** **n = 1,763**	**p-value**
Age [Mean (SD)]	54.9 (11.9)	57.3 (12.2)	57.7 (12.2)	56.8 (12.0)	<0.001
Gender (%)					
Male	52.6	52.1	48.7	36.8	<0.001
Female	47.4	47.9	51.3	63.2	
Race/Ethnicity (%)					
Non-Hispanic White	77.8	76.8	75.2	73.5	<0.001
Non-Hispanic Black	7.4	8.5	9.0	13.7	
Other	14.8	14.7	15.8	12.8	
BMI[1] [Mean (SD)]	25.5 (4.4)	27.9 (5.1)	29.7 (5.7)	32.4 (7.8)	<0.001
Education (%)					
Less than High School	15.5	17.8	22.2	23.6	<0.001
High School Graduate or Equivalent	25.7	24.6	27.1	26.7	
More than High School	58.8	57.6	50.7	49.8	
Smoking Status (%)					
Never Smoker	51.2	50.9	48.5	46.1	<0.001
Former Smoker	32.4	32.4	29.7	28.9	
Current Smoker	16.4	16.8	21.8	25.0	
Alcohol Intake (%)					
0 drinks/week	28.7	34.9	37.3	41.3	<0.001
< 1 drink per week	37.2	35.6	36.1	37.3	
> 1 drink per week	34.1	29.6	26.6	21.4	
Phytoestrogens (ng/mL) [Mean (SD)]					
Total Phytoestrogens	2266.3 (6222.8)	1613.0 (3009.8)	1549.6 (3665.9)	1243.2 (4159.3)	<0.001
Total Isoflavones	908.6 (3436.7)	639.8 (2152.9)	562.4 (2323.5)	487.5 (1601.8)	<0.001
Genistein	226.5 (965.5)	166.6 (720.0)	134.5 (615.2)	127.3 (407.0)	<0.001
Daidzein	481.5 (2028.9)	338.5 (1267.9)	295.6 (1335.9)	253.6 (823.5)	<0.001
Equol	80.0 (717.4)	51.4 (391.5)	54.1 (445.5)	48.9 (407.4)	0.24
O-desmethylangolensin	123.8 (694.5)	85.7 (408.1)	80.6 (510.1)	60.6 (463.2)	0.004
Total Lignans	1357.7 (4144.9)	973.3 (1912.4)	987.2 (2688.9)	755.7 (2166.3)	<0.001
Enterodiol	161.0 (602.6)	119.7 (335.9)	174.0 (1087.4)	187.8 (1800.4)	0.27
Enterolactone	1196.7 (3864.2)	853.7 (1730.2)	813.3 (1967.2)	568.2 (1057.0)	<0.001

[1] Body Mass Index

Table 12: Multiple linear regression analysis of serum C-reactive protein in relation to urinary excretion of total and individual phytoestrogens in the continuous National Health and Nutrition Examination Survey, 1999-2010

	C-Reactive Protein (mg/L)		
Phytoestrogens (ng/mL)	**Model 1**	**Model 2**	**Model 3**
Total Phytoestrogens			
β (95% CI)	-0.17 (-0.19, -0.15)	-0.19 (-0.22, -0.17)	-0.11 (-0.13, -0.09)
p-value	<0.001	<0.001	<0.001
Isoflavones			
β (95% CI)	-0.07 (-0.09, -0.05)	-0.08 (-0.10, -0.06)	-0.05 (-0.07, -0.04)
p-value	<0.001	<0.001	<0.001
Genistein			
β (95% CI)	-0.05 (-0.07, -0.03)	-0.06 (-0.07, -0.04)	-0.04 (-0.05, -0.02)
p-value	<0.001	<0.001	<0.001
Daidzein			
β (95% CI)	-0.05 (-0.07, -0.04)	-0.06 (-0.08, -0.04)	-0.04 (-0.06, -0.03)
p-value	<0.001	<0.001	<0.001
Equol			
β (95% CI)	-0.04 (-0.07, -0.02)	-0.06 (-0.08, -0.03)	-0.03 (-0.05, -0.01)
p-value	<0.001	<0.001	0.003
O-desmethylangolensin			
β (95% CI)	-0.05 (-0.06, -0.04)	-0.06 (-0.07, -0.05)	-0.04 (-0.05, -0.03)
p-value	<0.001	<0.001	<0.001
Lignans			
β (95% CI)	-0.13 (-0.15, -0.11)	-0.14 (-0.16, -0.13)	-0.08 (-0.10, -0.06)
p-value	<0.001	<0.001	<0.001
Enterodiol			
β (95% CI)	-0.07 (-0.08, -0.05)	-0.08 (-0.10, -0.06)	-0.04 (-0.05, -0.02)
p-value	<0.001	<0.001	<0.001
Enterolactone			
β (95% CI)	-0.11 (-0.12, -0.09)	-0.12 (-0.13, 0.10)	-0.07 (-0.08, -0.05)
p-value	<0.001	<0.001	<0.001

Model 1: Adjusted for creatinine level
Model 2: Further adjusted for age, gender, and race
Model 3: Further adjusted for education, BMI, smoking status, and alcohol intake

Table 13: ORs (95% CIs) for high concentrations of C-reactive protein (CRP) (>3mg/L) in relation to quartiles of urinary concentrations of total and individual phytoestrogens (ng/mL) in the continuous National Health and Nutrition Examination Survey, 1999-2010

	Quartile of Urinary Phytoestrogens				
Phytoestrogens (ng/mL)	**Q1**	**Q2**	**Q3**	**Q4**	**p-trend**
Total Phytoestrogens					
Subjects with CRP ≥ and < 3mg/L	780 / 1017	744 / 1055	633 / 1163	542 / 1254	
Model 1	Reference	0.88 (0.77 - 1.00)	0.66 (0.57 - 0.75)	0.50 (0.43 - 0.58)	<0.001
Model 2	Reference	0.83 (0.72 - 0.95)	0.60 (0.52 - 0.70)	0.45 (0.39 - 0.53)	<0.001
Model 3	Reference	0.88 (0.76 - 1.02)	0.67 (0.58 - 0.78)	0.63 (0.53 - 0.73)	<0.001
Isoflavones					
Subjects with CRP ≥ and < 3mg/L	718 / 1078	696 / 1104	671 / 1124	614 / 1183	
Model 1	Reference	0.90 (078 - 1.03)	0.83 (0.73 - 0.96)	0.72 (0.62 - 0.83)	<0.001
Model 2	Reference	0.89 (0.77 - 1.02)	0.81 (0.71 - 0.94)	0.69 (0.60 - 0.79)	<0.001
Model 3	Reference	0.86 (0.74 - 1.00)	0.75 (0.65 - 0.88)	0.73 (0.63 - 0.86)	0.002
Genistein					
Subjects with CRP ≥ and < 3mg/L	695 / 1106	701 / 1093	673 / 1124	629 / 1167	
Model 1	Reference	0.98 (0.85 - 1.12)	0.90 (0.78 - 1.03)	0.80 (0.70 - 0.92)	0.001
Model 2	Reference	0.96 (0.84 - 1.11)	0.89 (0.78 - 1.03)	0.78 (0.67 - 0.90)	<0.001
Model 3	Reference	0.95 (0.82 - 1.11)	0.85 (0.73 - 0.99)	0.80 (0.69 - 0.94)	0.010
Daidzein					
Subjects with CRP ≥ and < 3mg/L	707 / 1094	710 / 1084	641 / 1156	641 / 1156	
Model 1	Reference	0.97 (0.85 - 1.11)	0.81 (0.71 - 0.93)	0.80 (0.70 - 0.92)	0.003
Model 2	Reference	0.95 (0.83 - 1.09)	0.80 (0.70 - 0.92)	0.77 (0.67 - 0.89)	0.001
Model 3	Reference	0.94 (0.81 - 1.09)	0.77 (0.66 - 0.90)	0.79 (0.67 - 0.92)	0.009
Equol					
Subjects with CRP ≥ and < 3mg/L	682 / 1077	667 / 1087	664 / 1094	616 / 1138	
Model 1	Reference	0.95 (0.83 - 1.09)	0.90 (0.78 - 1.03)	0.78 (0.67 - 0.90)	<0.001
Model 2	Reference	0.94 (0.82 - 1.07)	0.90 (0.78 - 1.03)	0.77 (0.66 - 0.89)	<0.001
Model 3	Reference	0.92 (0.80 - 1.07)	0.91 (0.78 - 1.06)	0.76 (0.65 - 0.89)	0.001
O-desmethylangolensin					
Subjects with CRP ≥ and < 3mg/L	743 / 1064	697 / 1041	604 / 1154	599 / 1168	
Model 1	Reference	0.93 (0.82 - 1.07)	0.73 (0.63 - 0.83)	0.71 (0.62 - 0.81)	<0.001
Model 2	Reference	0.93 (0.81 - 1.07)	0.71 (0.62 - 0.82)	0.67 (0.58 - 0.77)	<0.001
Model 3	Reference	0.91 (0.78 - 1.05)	0.71 (0.61 - 0.82)	0.72 (0.62 - 0.84)	0.002
Lignans					
Subjects with CRP ≥ and < 3mg/L	789 / 1008	727 / 1073	639 / 1157	544 / 1251	
Model 1	Reference	0.85 (0.74 - 0.97)	0.68 (0.59 - 0.77)	0.50 (0.44 - 0.58)	<0.001
Model 2	Reference	0.80 (0.70 - 0.92)	0.63 (0.55 - 0.72)	0.46 (0.40 - 0.53)	<0.001
Model 3	Reference	0.87 (0.75 - 1.00)	0.73 (0.63 - 0.85)	0.64 (0.54 - 0.75)	<0.001
Enterodiol					
Subjects with CRP ≥ and < 3mg/L	738 / 1058	649 / 1147	659 / 1137	649 / 1144	
Model 1	Reference	0.79 (0.69 - 0.91)	0.78 (0.68 - 0.90)	0.75 (0.65 - 0.86)	0.004
Model 2	Reference	0.77 (0.67 - 0.88)	0.76 (0.66 - 0.87)	0.69 (0.60 - 0.79)	<0.001
Model 3	Reference	0.87 (0.75 - 1.01)	0.81 (0.70 - 0.95)	0.87 (0.75 - 1.02)	0.37
Enterolactone					
Subjects with CRP ≥ and < 3mg/L	805 / 1004	720 / 1075	651 / 1141	522 / 1269	
Model 1	Reference	0.83 (0.73 - 0.95)	0.69 (0.61 - 0.79)	0.48 (0.41 - 0.55)	<0.001
Model 2	Reference	0.80 (0.70 - 0.92)	0.66 (0.58 - 0.76)	0.45 (0.39 - 0.51)	<0.001
Model 3	Reference	0.83 (0.72 - 0.96)	0.76 (0.65 - 0.88)	0.59 (0.51 - 0.69)	<0.001

Model 1: Adjusted for creatinine level

Model 2: Further adjusted for age, gender, and race

Model 3: Further adjusted for education, BMI, smoking status, and alcohol intake

Chapter 5

General Discussion, Conclusions, and Perspectives

The results of the analyses of these three studies provided evidence that the intake of both total and individual phytoestrogens alters the risk of cancer and cardiovascular disease. The results of the first study using the NHANES data suggested that increased urinary concentration of total lignans were associated with a reduced risk of cardiovascular disease mortality. In addition, increased concentrations of both total lignans and enterolactone were associated with a reduced risk of all-cause mortality. Conversely, increased concentrations of total isoflavones and daidzein were associated with an increased risk of both cardiovascular and all-cause mortality. The results of the second study using the PLCO data suggested that higher dietary intake of isoflavones, genistein, daidzein, and glycitein were associated with an increased risk of development of advanced prostate cancer. Conversely, higher dietary intake of genistein was associated with a reduced risk of non-advanced, and total prostate cancer. Finally, the results of the third study using the NHANES data suggested that higher urinary concentrations of total and individual phytoestrogens were associated with reduced concentrations of CRP, with the largest reduction observed in total phytoestrogens, lignans, and enterolactone. Increased CRP levels have been associated with an increased risk of certain chronic conditions, such as cardiovascular disease (229, 230), whereas increased phytoestrogen intake has been associated with a decreased risk of these conditions (168, 169).

The results of these studies added to the knowledge of modifiable risk factors that may either increase or decrease the risk of prostate cancer or cardiovascular disease. The identification of modifiable risk factors is important for these types of chronic conditions that affect such a large population in the US and the world. Two results in particular from this study have the potential to impact these diseases. The first of these most significant results was that total lignans was significantly associated with a decreased risk of cardiovascular disease mortality. This finding represents a modifiable risk factor that may be protective against the condition responsible for the highest number of deaths in the US (1). Although other phytoestrogens (isoflavones and daidzein) were associated with an increased risk of cardiovascular disease mortality, these types of phytoestrogens are found in the highest concentrations in different foods (43) compared to lignans (126). The second most significant result was that the intake of isoflavones, genistein, daidzein, and glycitein were associated with an increased risk of the development of advanced prostate cancer. If these results are confirmed in additional studies, the identification of these phytoestrogens as a factor that increases the risk of advanced prostate cancer may be the most valuable contribution of these studies.

There are several strengths of these studies that added to the validity of the findings. The datasets that were used in the analyses of all of the studies were relatively large and in the case of the NHANES data, nationally representative of the US population. The PLCO data was large enough that the cases of prostate cancer could be divided into either non-advanced or advanced cases, which allowed for unique analyses of the data that has not been attempted on a US population in the past. Many similar studies conducted in the past consisted of small case-control or cross-sectional designs. The subjects who

participated in both the NHANES and the PLCO were followed prospectively, allowing for a temporal element to the data analyses. The prospective nature of the data also largely excluded the possibility of reverse causality between phytoestrogens and the outcomes of interest.

Results from these current studies showed both similarities and differences to previous published literature. Although experimental or epidemiologic data are scarce on the association between phytoestrogens and cardiovascular disease, there have been a small number of studies that show certain phytoestrogens may be associated with a decreased risk of cardiovascular disease (167-169). Although other studies have shown a null association between phytoestrogens and cardiovascular disease (170-172), these dissimilar results may be because most previous studies have focused on small, specific populations such as postmenopausal women, or the differences in the outcome measures between previous studies and this first study.

Many previous studies have suggested that dietary intake of genistein or other isoflavones may exert different effects on specific types of cancer (159, 160, 163), however the current first study showed no association with cancer mortality for any of the phytoestrogen measurements. Although the risk of certain cancers may be altered by the intake of phytoestrogens, this cannot be assumed for all variations of this disease. Given the small number of total cancer deaths (n=79) in the first study, it was not possible to examine cancer-specific associations with total and individual phytoestrogens, which may account for the differences that observed in previous studies.

The results of the second study showed both a significant increased risk between of advanced prostate cancer, and a significant decreased risk of non-advanced and total

prostate cancer associated with dietary genistein. Most previous studies have suggested that dietary intake of genistein is inversely associated with prostate cancer (159, 182); however, one experimental study also suggested that genistein may promote tumor progression in advanced prostate cancer (84). Although it remains unclear why genistein simultaneously increased the risk of advanced prostate cancer and decreased the risk of non-advanced prostate cancer due to a lack of previous experimental and epidemiologic studies, there may be a biologic plausibility for the increased risk of advanced prostate cancer observed in the second study. It has been hypothesized that estrogen may play a role in prostate carcinogenesis due to the possible mutagenic effects of estrogen metabolites (205). Phytoestrogens can induce estrogenic responses in the body due to their structural similarity to 17β-estradiol (12). Additionally, increased risk of advanced prostate cancer has been observed in patients treated with finasteride, which inhibits the conversion of testosterone to dihydrotestosterone, causing an increase of estrogen levels (206). According to the results of these previous studies, it is reasonable to assume that an increased intake of phytoestrogens exhibiting increased estrogenic activity would also be associated with an increased risk of advanced prostate cancer.

The results of this third study showed through both linear and logistic regression that urinary concentrations of phytoestrogens were significantly inversely associated with CRP levels. These results were consistent with several previous studies that showed increased intake of dietary phytoestrogens were associated with a decrease in CRP levels (235-237). However, other studies observed that phytoestrogens did not alter CRP levels (231, 238). Previous studies have shown that CRP levels may be reduced in the presence of antioxidants (239, 240) and phytoestrogens have been shown to have antioxidant

properties (241, 242). Additional studies have shown that higher levels of circulating estrogens are associated with increased levels of CRP (243, 244). It is possible that CRP levels in the third study were reduced either due to the antioxidant properties of phytoestrogens, or the competitive binding of phytoestrogens to estrogen receptors, resulting in reduced effects of natural, more biologically potent estrogens on CRP.

In summary, dietary intake of certain phytoestrogens were found to significantly modulate prostate cancer risk and cardiovascular disease mortality. It is important to determine modifiable risk factors that may affect the risk of chronic conditions and therefore increase opportunities for primary prevention. The observed increased risk between total and individual isoflavones and advanced prostate cancer may offer an innovative, practical avenue for the prevention of biologically relevant manifestations of this disease by decreasing the intake of soy products, legumes, and chickpeas. In addition, it is possible that the associations between phytoestrogens and cardiovascular disease may be in part mediated through the influence of phytoestrogen intake on circulating levels of C-reactive protein. Since phytoestrogens are biologically weaker compared with natural or pharmacological estrogens, an increase in the dietary intake of foods containing these compounds may decrease the risk of cardiovascular disease through competitive binding to estrogen receptors.

Further research is needed to confirm the results that were found in these studies. The nature of the data in the first study did not allow for the performance of stratified analyses by individual types of cancer or cardiovascular disease. Updated NHANES data with a longer follow-up period may be able to provide new insights into the associations between phytoestrogens and specific variations of these chronic conditions. In the

second study, phytoestrogen levels in the PLCO data were estimated using a food frequency questionnaire. Nutritional data collected in this manner is subject to recall bias and the levels of certain phytoestrogens may be inadequately represented in food composition databases. Additionally, this method of phytoestrogen measurement is not able to quantify phytoestrogen metabolites that are produced in the intestinal bacteria, such as equol and O-desmethylangolensin (71). Future research that is able to investigate the effects of total and individual phytoestrogens measured through urinary biomarkers on both advanced and non-advanced prostate cancer could help to solidify the findings. The use of urinary biomarkers could also quantify the risk between lignans and prostate cancer. In the first study, lignans showed a protective effect associated with cardiovascular disease mortality, whereas isoflavones and genistein were associated with an increased risk. The second study showed a similar increased risk between total and individual isoflavones and advanced prostate cancer, but data on lignans were not available. It would be interesting to observe the associations between lignans and advanced prostate cancer, as lignans and isoflavones originate from different food sources. Finally, the design of the third study was cross-sectional in nature, which only allows for the capture of information at one point in time. These results may not accurately reflect the usual intake of phytoestrogens or usual plasma CRP levels. A future study that consists of repeated measures or a longitudinal design could add valuable information to the relationship between phytoestrogens and CRP.

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inflammatory biomarkers and proinflammatory cytokines in middle-aged men and women. Metabolism. 2002;51:919-24.

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postmenopausal women (from the Estrogen in the Prevention of Atherosclerosis Trial). American Journal of Cardiology. 2008;101:1131-33.

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CURRICULUM VITAE

Michael Kent Reger

Education

- **Ph.D.** Epidemiology 2009-2014
  Minor: Health Informatics
  - Indiana University, Indianapolis, IN 2010-2014
  - University of Iowa, Iowa City, IA 2009-2010
- **M.P.H.** Epidemiology, Indiana University, Indianapolis, IN 2006-2009
- **B.S.** Health Science, Purdue University, West Lafayette, IN 2000-2005
  Minors: Biology and Organizational Leadership

Research Interests

My research is focused on modifiable risk factors, particularly nutritional factors, associated with the risk of specific chronic conditions. Although knowledge of the biological mechanisms of chronic diseases has expanded greatly due to the emergence of more accessible genetic information and increased experimental studies, there is still a great deal of data that remains underutilized, with many potential risk factors left unexplored by epidemiologists. My work to this point has involved the analysis of existing national datasets using rigorous statistical tests to determine the association between one of these underexplored modifiable risk factors, phytoestrogens, and the risk of cancer or cardiovascular disease. The principal goal of epidemiology remains the primary prevention of disease, and I am committed to the identification of factors that may assist in the alleviation of the disease burden resulting from these chronic conditions. Moving forward, I am interested in utilizing additional data sources and statistical tests to explore nutritional and other risk factors and their association with a variety of chronic conditions.

In the immediate future my research plans include:

- Utilizing the PLCO data to determine the association between phytoestrogens and other hormone related cancers (breast, colorectal, and ovarian cancers)
- Utilizing updated mortality data from NHANES to further explore the relationship between phytoestrogens and cardiovascular disease or cancer

My longer term goals are focused on the identification of genetic risk factors that may influence the association between phytoestrogens or other nutrients and chronic disease. The identification of both genetic and nutritional factors associated with an increased risk of chronic conditions will assist in the determination of the highest risk populations. With this knowledge, health care providers and other entities will be better able to focus their efforts and resources, thereby increasing the opportunity for primary disease prevention.

Awards

- Indiana University Fairbanks School of Public Health Doctoral Dissertation Fellowship 2013
- Indiana University Center for Urban Health Fellowship 2010

Skill Set

- **Data Analysis**

Extensive experience with both SAS and SPSS statistical analysis packages analyzing large datasets, including Behavioral Risk Factor Surveillance System (BRFSS), Youth Risk Behavior Surveillance System (YRBSS), National Health and Nutrition Examination Survey (NHANES), and Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO).

- **Writing**

Experience in writing numerous manuscripts, internal review board (IRB) documentation, and grants.

- **Community Assessment**

Experience with survey development, data collection, data analysis, and writing the final report.

- **Teaching**

Experience with course development, lecture preparation, and lecture delivery.

Professional Experience

- **Research Experience**

Indiana University, Doctoral Dissertation Fellow 2013-2014
Richard M. Fairbanks School of Public Health

- Principle Investigator and primary author for three nutritional based epidemiology manuscripts
- Extensive data and analysis on large datasets: NHANES and PLCO, using both SAS and SPSS

Indiana University, Graduate Research Assistant 2012-2013
Richard M. Fairbanks School of Public Health

- Assisted on projects concerning Vitamin K and Prostate Cancer, Prenatally Diagnosed Congenital Anomalies, Community Assessments, and Exercise Physician Survey for the American College of Sports Medicine
- Tasks included: Data analysis, Examining spatial variation using ArcGIS, Database updates and analysis, Literature reviews, and Poster composition and presentation

Indiana University, Center for Urban Health Fellow 2010-2011
School of Science

- Executed a selected review of literature (several hundred manuscripts) of three modifiable risk factors (physical activity, smoking, and obesity) pertaining to urban populations
- Performed preliminary data analysis utilizing the BRFSS to discover differences between the three modifiable risk factors among the urban and rural populations of Indiana
- Began preliminary analysis on a new data set pertaining to recidivism among at risk youth utilizing data from the Indiana Juvenile Mental Health Screening Project – a statewide collaboration to initiate screening within youth detention centers

University of Iowa, Graduate Research Assistant 2009-2010
College of Public Health

- GRA for study of Vitamin D blood levels and risk of Malignant Melanoma (ongoing)
- Organized all study subject appointments and materials
- Performed preliminary data analysis using SAS
- Conducted literature reviews
- Pooled data for meta-analysis
- GRA for evaluation of *Community Circle of Care* program based in Des Moines, Iowa
- Performed data analysis on preliminary survey data to determine program outcomes using SPSS
- Wrote reports to identify outcomes to program staff

Indiana University, Research Assistant 2008-2009
School of Medicine Bowen Research Center

- Performed data analysis with SPSS for a collaboration project with the Indiana State Department of Health to determine the 10 year obesity trend for the state of Indiana using data from the BRFSS
- Performed data and projection analysis for the Indiana Physician's/Nurse's/Oral Health Professional's Report by analyzing trends from survey data using SPSS statistical software
- Designed data maps using ArcMap GIS

- **Teaching Experience**
  Indiana University

  Graduate Level
  - PBHL E715 – Design and Implementation of Observational Studies (guest lecturer) 2010
  - PBHL E775 – Doctoral Research Seminar (guest lecturer) 2013

  Undergraduate Level
  - PBHL A322 – Principles of Epidemiology (primary course instructor – 9 credit hours) 2011-2012

Publications

Peer Reviewed Publications

1. Humbert L, Saywell RM, Zollinger TW, Priest CF, **Reger MK,** Kochhar K. "The Effect of Pregnancy Intention on Important Maternal Behaviors and Satisfaction with Care in a Socially and Economically At-Risk Population." Maternal and Child Health Journal (MCHJ) 2010.
2. Kochhar K, Saywell RM, Zollinger TW, Mandzuk CM, Haas DM, Howell LK, Sevilla JM, **Reger MK.** "Herbal Use among Hispanic Women and Hispanic Women with a History of Pregnancy or Breast Feeding: Are Physicians Informed?" Hispanic Health Care International (HHCI) 2010; 8(1).

Other Publications

1. Hutber, A.C., Williams, J., **Reger, M.K.,** Seyffarth, C.G., Zollinger, T.W., "Physician Attitudes about Patient Physical Activity Counseling and Referrals." For the American College of Sports Medicine, *Exercise is Medicine* © program, December 2013.
2. Hutber, A.C., Williams, J., Seyffarth, C.G., **Reger, M.K.,** Zollinger, T.W. "Physician Attitudes about Patient Physical Activity Counseling and Referrals." American Public Health Association 141st Annual Meeting and Exposition, Boston, November 2013 (poster).
3. **Reger, MK.,** Harris, E., Wessel, J., Litton, C., Schubert, F., Golichowski, A., & Lee, MJ. "Analysis of Spatial Variation in Prenatally Diagnosed Congenital Anomalies in Indiana." The Society for Maternal-Fetal Medicine Annual Meeting, San Francisco, 2013. (Poster).
4. **Reger, M.K.,** Mahoui, M., Zollinger, T.W. "Good Samaritan Hospital Community-Based Needs Assessment: Summary Report." Good Samaritan Hospital. May 2013.
5. **Reger, MK.,** Harris, E., Wessel, J., Litton, C., Schubert, F., Golichowski, A., & Lee, MJ. "Analysis of Spatial Variation in Prenatally Diagnosed Congenital Anomalies in Indiana." First Annual Public Health Research and Service Symposium, Indianapolis, 2012. (Poster).
6. Humbert, L., Saywell, R.M., Zollinger, T.W., Priest, C.F., **Reger, M.K.,** Kochhar, K. "The Effects of Pregnancy Intention on Maternal Behaviors and Satisfaction with Care." 2011 National Perinatal Association Conference, Louisville, October 2011 (poster).

7. Humbert, L., Saywell, R.M., Zollinger, T.W., Priest, C.F., **Reger, M.K.,** Kochhar, K. "The Effect of Pregnancy Intention on Important Behaviors during Pregnancy and Satisfaction with Care in a Socially At-risk Population." 20th Anniversary CityMatCH Conference: Urban Maternal and Child Health Leadership, Chicago, September 2010.
8. Zollinger, TW. Kochhar, K., **Reger, MK.,** Alyea, JM. "2007 Indiana Registered nurse Re-Licensure Survey Report." Indiana Center for Health Workforce Studies. 2010.
9. Allen DI, Zollinger, TW, Kochhar, K., **Reger, MK,** Chowdhury, S. "Responses to the 2003, 2005, and 2007 Indiana Physician Surveys." For the Indiana Area Health Education Centers Program, April 2009.

Manuscripts Accepted for Revision

1. **Reger, M.K.,** Zollinger, T., Liu, Z., Jones, J., & Zhang, J. "Urinary excretion of phytoestrogens and the risk of cancer mortality, cardiovascular mortality, and all-cause mortality in the Continuous National Health and Nutrition Examination Survey."

Manuscripts in Process

1. **Reger, M.K.,** Zollinger, T., Liu, Z., Jones, J., & Zhang, J. "Dietary intake of phytoestrogens and the risk of total and advanced prostate cancer in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO)."
2. **Reger, M.K.,** Harris, E., Wessel, J., Litton, C., Schubert, F., Golichowski, A., Lee, M.J. "Analysis of Spatial Variation in Prenatally Diagnosed Congenital Anomalies in Indiana."
3. **Reger, M.K.,** Zollinger, T., Liu, Z., Jones, J., & Zhang, J. "Association between urinary phytoestrogens and C-reactive protein in the continuous National Health and Nutrition Examination Survey."

Graduate Course Work

- **Indiana University,** Richard M. Fairbanks School of Public Health

Fundamentals of Epidemiology, Philosophies and Principles of Health Education, Biostatistics for Public Health I & II, US Healthcare Policies and Ethical Challenges, Environmental Health Science in Public Health, Epidemiologic Research Methods, Advanced Epidemiology, Patient Centered Outcomes Research, Cancer Epidemiology, Introduction to Informatics, Infectious Disease Epidemiology, Methods for Research on Social and Behavioral Dimensions in Public Health, Design and Implementation of Observational Studies, Molecular Epidemiology, Instrumentation Development and Measurement, Analysis and Interpretation of Observational Studies, Applied Multivariate Statistical Methods, Foundations of Health Informatics, Qualitative Inquiry and Research Methods, Pharmacoepidemiology, Clinical Trials, Clinical Information Systems, Applied Spatial Statistics, Clinical Decision Support Systems, Foundations in Public Health Informatics, Business of Health Informatics

- **University of Iowa,** College of Public Health

Epidemiology Data Analysis, Principles of Epidemiology, Introduction to Biostatistics, Introduction to Human Pathology, Design and Analysis of Biomedical Studies, Epidemiology Advanced Methods, Epidemiology of Chronic Diseases, Cancer Epidemiology and Control